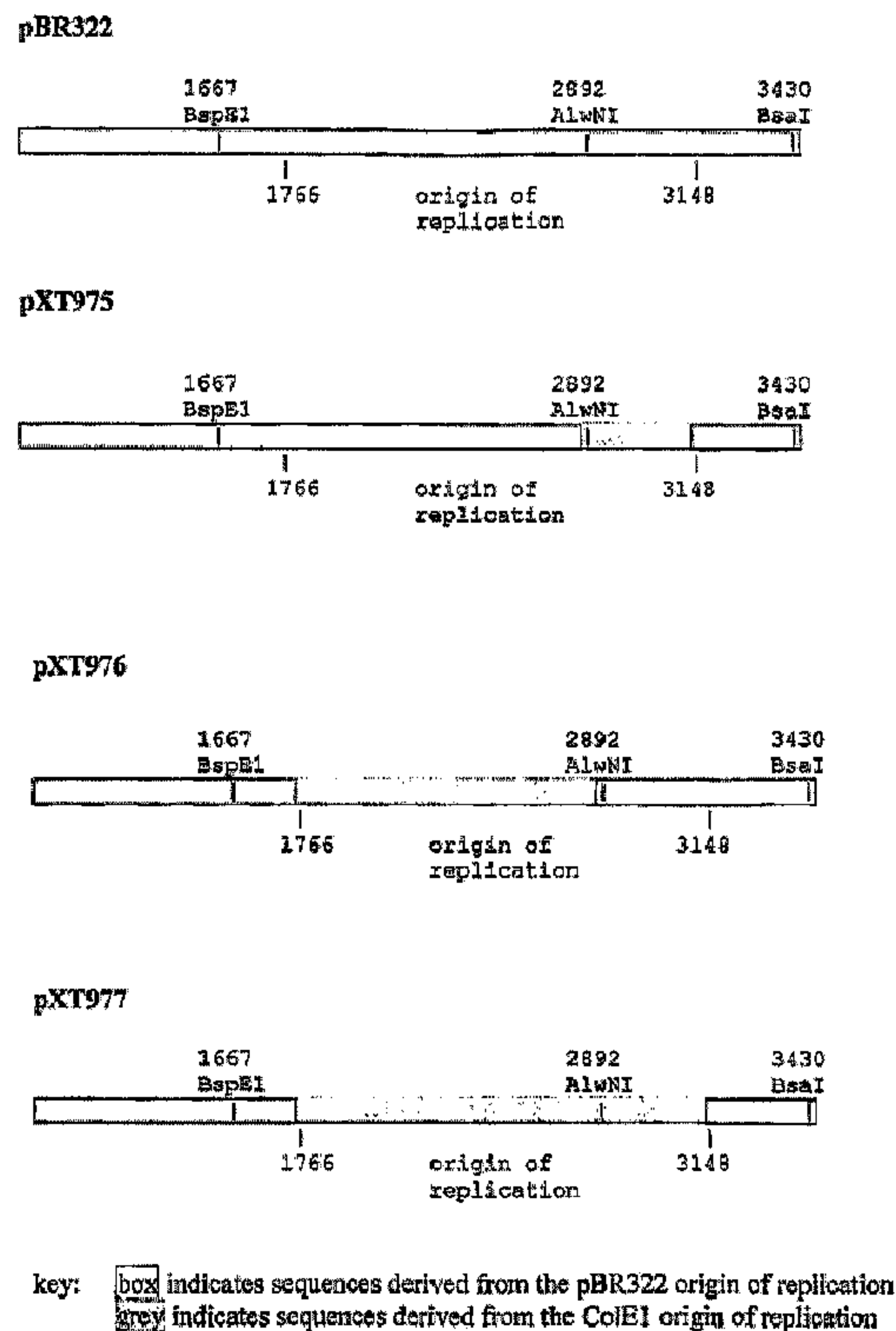




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(72) **Inventeurs/Inventors:**
BOGOSIAN, GREGG, US;
FRANTZ, JULIA P., US;
SMITH, HONG Q., US
(73) **Propriétaire/Owner:**
MONSANTO TECHNOLOGY LLC, US
(74) **Agent:** OSLER, HOSKIN & HARCOURT LLP

(54) **Titre : ORIGINE TRANSFERABLE HYBRIDE DE PLASMIDES A REPLICATION**
(54) **Title: HYBRID PORTABLE ORIGIN OF REPLICATION PLASMIDS**



(57) **Abrégé/Abstract:**

The invention relates to modifying plasmid origins of replication to create hybrid origins of replication containing nucleotide sequences from more than one plasmid. The invention also relates to a modified origin of replication cassette that is portable or

(57) Abrégé(suite)/Abstract(continued):

exchangeable due to the creation of multiple cloning sites flanking the origin of replication. Methods and plasmids for use in exchanging origins of replication are disclosed. Such modified or hybrid plasmids provide useful cloning tools that allow for regulation of the level of expression of a desired protein.

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(71) Applicant (for all designated States except US): **MON-SANTO TECHNOLOGY LLC** [US/US]; 800 North Lindbergh Boulevard, St. Louis, MO 63167 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **BOGOSIAN, Gregg** [US/US]; 374 Herworth Court, Clarkson Valley, MO 63005 (US). **O'NIEL, Julia, P.** [US/US]; 24 Hilliard Road, Glen-dale, MO 63122 (US). **SMITH, Hong, Q.** [US/US]; 13501 Cedar Bridge Road, St. Louis, MO 63141 (US).

(74) Agent: **KAMMERER, Patricia, A.**; HOWREY, LLP, 2941 Fairview Park Drive, Box 7, Falls Church, VA 22042 (US).

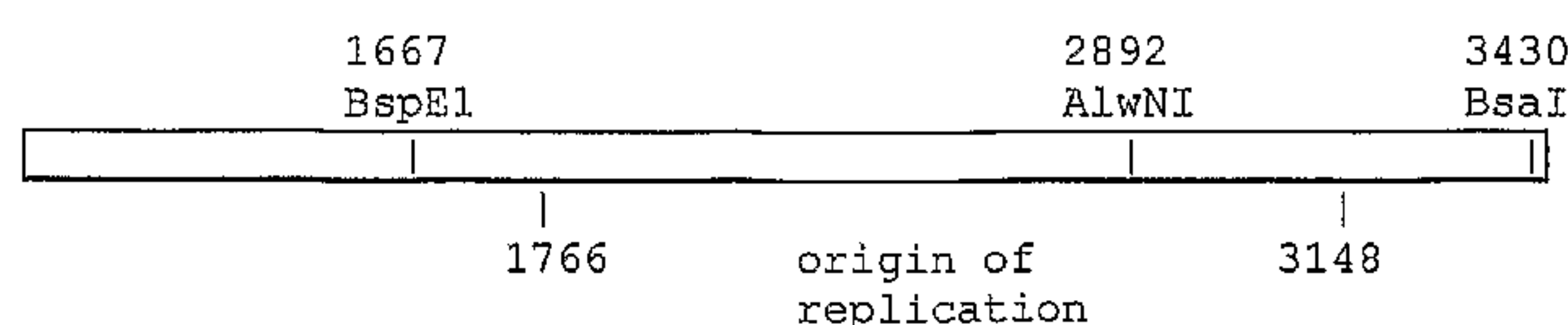
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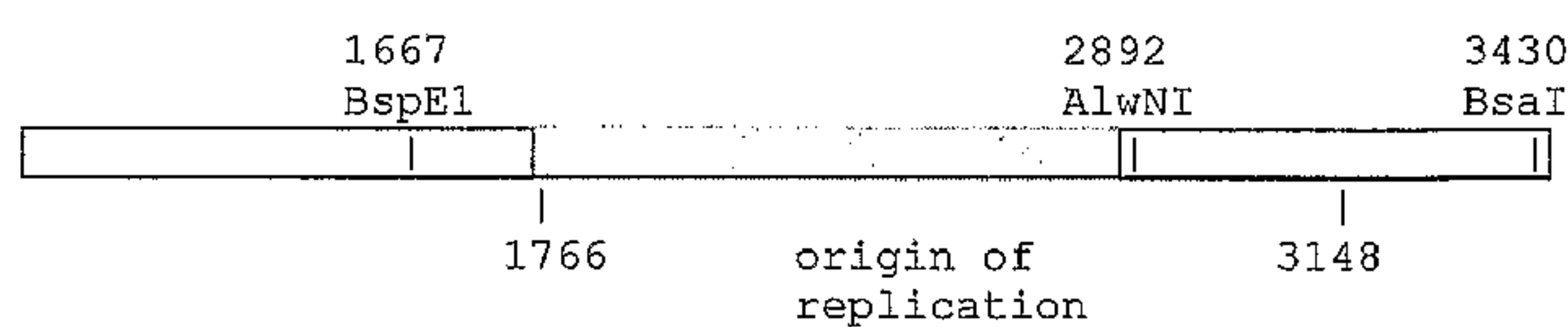
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(54) Title: HYBRID PORTABLE ORIGIN OF REPLICATION PLASMIDS

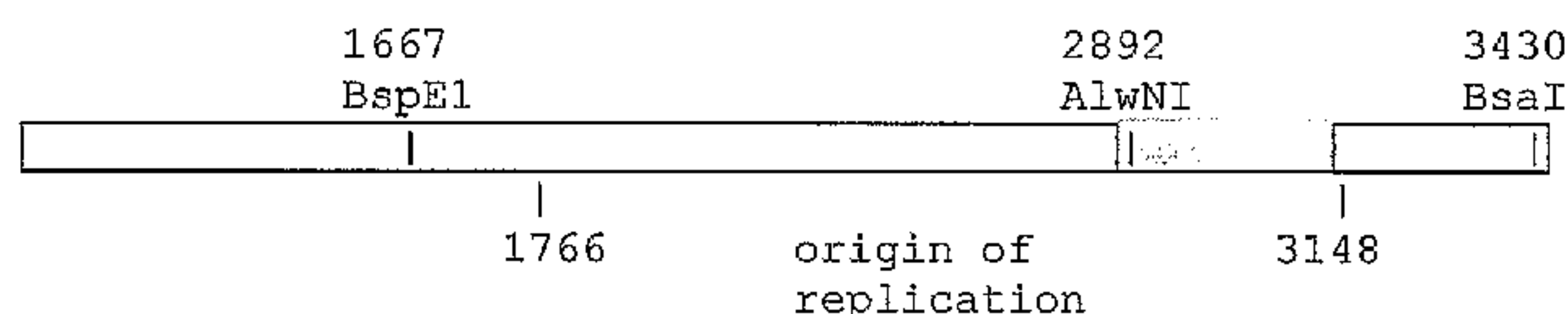
pBR322



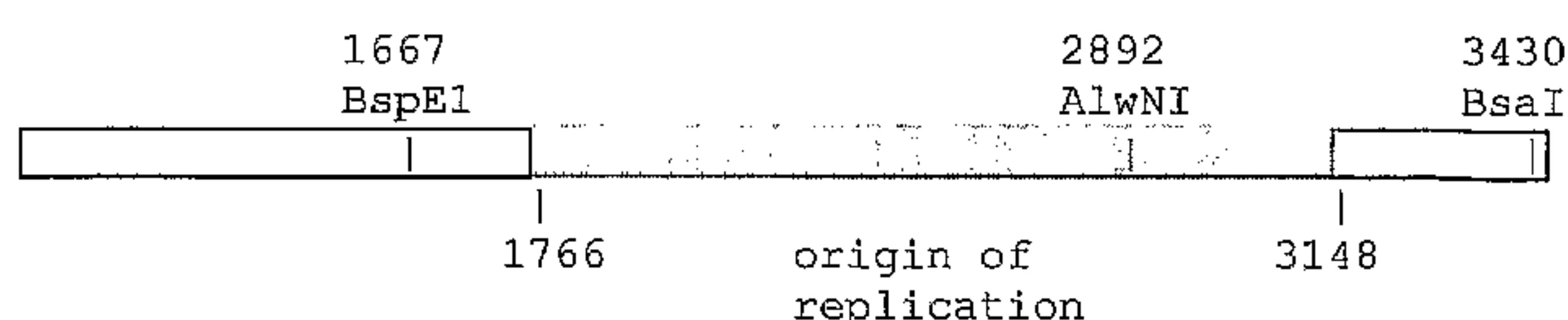
pXT975



pXT976



pXT977



key: indicates sequences derived from the pBR322 origin of replication
 indicates sequences derived from the ColE1 origin of replication

(57) Abstract: The invention relates to modifying plas-mid origins of replication to create hybrid origins of repli-cation containing nucleotide sequences from more than one plasmid. The invention also relates to a modified ori-gin of replication cassette that is portable or exchangeable due to the creation of multiple cloning sites flanking the origin of replication. Methods and plasmids for use in ex-changing origins of replication are disclosed. Such mod-ified or hybrid plasmids provide useful cloning tools that allow for regulation of the level of expression of a desired protein.

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HYBRID PORTABLE ORIGIN OF REPLICATION PLASMIDS

FIELD OF THE INVENTION

[0001] The invention relates to modifying plasmid origins of replication and, more specifically, to a modified portable origin of replication for plasmids pBR322 or pACYC184. In particular, methods and plasmids for use in exchanging origins of replication are disclosed.

DESCRIPTION OF RELATED ART

[0002] Many species of bacteria contain small circular extrachromosomal genetic elements, known as plasmids. Plasmids replicate independently of the bacterial cell's chromosome. They are usually small, circular, double-stranded molecules, found in all types of bacteria (perhaps all species, although not all strains, of bacteria). The number of copies (i.e., the copy number) varies from plasmid type to plasmid type, and a cell can have more than one type of plasmid. Plasmids contain genes that are non-essential, but often beneficial, to the bacterium. Common genes found in plasmids include those encoding plasmid replication (i.e., the origin of replication) and cellular maintenance, antibiotic resistance, bacteriocin production, sex determination, and other cellular functions (Kornberg and Baker, *DNA Replication*, 2nd ed. (1991)).

[0003] A number of plasmids are known in the art such as pBR322, pMB1, p15A, pACYC184, pACYC177, ColE1, pBR3286, p1, pBR26, pBR313, pBR327, pBR328, pPIGDM1, pPVUI, pF, pSC101 and pC101p-157.

[0004] Many microbial plasmid expression vectors are derivatives of the pBR322 plasmid. The plasmid pBR322 is one of the most commonly used *Escherichia coli* cloning vectors, a map of which is shown in Figure 1. pBR322 is 4361 base pairs in length and contains the following genetic elements: (1) the replicon responsible for the replication of plasmid (the origin of replication is from plasmid pMB1); (2) the *rop* gene coding for the Rop protein, which promotes conversion of the unstable RNA I – RNA II complex to a stable complex and serves to decrease copy number; (3) the *bla* gene, coding for beta-lactamase that confers resistance to ampicillin; and (4) the *tetR* gene, encoding the tetracycline resistance protein. The complete nucleotide sequence of the plasmid pBR322 has been determined, revealing several unique restriction sites useful for the cloning of DNA fragments.

[0005] The indicated *rep* region is sufficient to promote replication. DNA replication initiates at position 2533 and proceeds in the direction indicated in Figure 1. Plasmids carrying the pMB1

and ColE1 replicons are incompatible, but they are fully compatible with those carrying the p15A replicon (such as the standard cloning vectors pACYC177 and pACYC184).

[0006] The plasmid pBR322 has several important advantages that have led to it being widely and successfully employed as the starting plasmid for constructing many expression vectors. The advantages include that pBR322 has been completely sequenced, has several unique restriction sites useful for cloning DNA fragments, is not self-transmissible, and is readily available.

[0007] However, there are a number of disadvantages associated with using pBR322. One of the important features of a plasmid is its copy number, which is set by the region on the plasmid called the origin of replication. The origin of replication on the plasmid pBR322 extends from numbered coordinates 1766 to 3148.

[0008] The copy number of a plasmid, that is, the average number of plasmid molecules per cell, is a fundamental plasmid characteristic that is determined and regulated by an array of plasmid genetic elements (reviewed in *The Biology of Plasmids*, David K. Summers, Blackwell Science, 1996; and in *Plasmid Biology*, Barbara E. Funnell and Gregory J. Phillips, American Society for Microbiology Press, 2004). Each specific plasmid has a characteristic plasmid copy number. Of the dozens of plasmids studied to date, specific plasmid copy numbers have been found to range from as few as one copy per cell to hundreds of copies per cell. The plasmid copy number has a number of important effects on the host cell. Often, over-expression of a gene of interest on a multicopy number plasmid can have toxic effects on a cell and make cloning the gene of interest difficult if not impossible. Under conditions where high copy number is undesirable, it is possible to utilize a lower copy number plasmid in these circumstances, such as pACYC184, which is considered a low copy number plasmid. In certain cases, it is desirable to utilize a plasmid with a high copy number, such as the pBluescript® series of vectors (available from Stratagene, La Jolla, California) that are modified ColE1 plasmids with a high copy number and other useful features.

[0009] One of the disadvantages of pBR322 is its copy number of about 20, placing it in the low copy number range. The origin of replication of pBR322 is derived from the plasmid pMB1 and is homologous to the origin of replication region on plasmid ColE1. Thus, while it is often stated that pBR322 has a ColE1 origin of replication, it actually has a pMB1 origin of replication.

[0010] Since the plasmid pBR322 has a low copy number, it is often necessary to utilize a different plasmid with a high copy number to generate large amounts of an over-expressed desired protein. In other instances, it is desirable to utilize a low copy number plasmid, in order to get expression of certain toxic proteins, where the host cell does not tolerate over-expression. Thus, it has previously been the case that if a plasmid with a significantly higher or lower plasmid copy number than that of pBR322 were desired for cloning purposes, a plasmid without the many useful features of pBR322 would have to be utilized. It would be a great benefit to provide a modified pBR322 plasmid that has some characteristics of the pMB1 ORI and some characteristics of another ORI such as the ORI from ColE1. It would also be of great benefit to provide a modified pBR322 plasmid that has easily exchangeable origin of replication, which could be utilized as an expression plasmid, and that otherwise remains unchanged. In this manner, one could still have the benefits of the pBR322 plasmid, and also have the flexibility of being able to change the origin of replication to any desired alternative origin of replication.

[0011] When there is a preference for using a particular plasmid, such as desired antibiotic resistance markers or restriction sites, it would be beneficial to be able to modify the origin of replication to obtain a desirable copy number. Previously, there has been no success modifying the origin of replication of pBR322 with a different plasmid origin of replication region because the pBR322 origin of replication region, 1766-3148, is not flanked by unique restriction sites. Beyond coordinate 3148, the first unique restriction site is located within the ampicillin resistance gene. Thus, it is not possible to simply "cut and paste" other origin of replications into the pBR322 plasmid backbone.

[0012] Thus, there exists a need for a modified pBR322 plasmids, containing a portable origin of replication, preferably flanked by multiple unique restriction sites known as multiple cloning sites (MCS), creating an easily exchangeable pBR322 origin of replication. Such modified pBR322 plasmids will provide useful cloning tools that allow for regulation of the level of expression of desired or target gene products.

[0013] A similar situation exists for several other plasmids, such as pACYC184, p1, plasmid F and pSC101. pACYC184, with its low copy number and its origin of replication region lacking unique restriction sites for facilitating exchange of the origin of replication region could be readily used if were modified with flanking MCS.

[0014] Bacteriophage P1 exists inside *E. coli* cells in the quiescent prophage state as an independent plasmid with a copy number of about 2-3 per cell, rather than being integrated into the *E. coli* chromosome like most other prophage. Thus, the origin of replication of the P1 genome can be employed as a plasmid origin of replication. The origin of replication of P1 contains genetic elements that provide for stable inheritance of the plasmid, making this origin of replication attractive for use as the basis of a stable 2-3 copy number plasmid cloning vector.

[0015] The plasmid F, with a plasmid copy number of about 1-2 per cell, is a plasmid of *E. coli* involved in genetic exchange. The origin of replication of the plasmid F contains genetic elements that provide for stable inheritance of the plasmid, making the plasmid F origin of replication attractive for use as the basis of a stable 1-2 copy number plasmid cloning vector.

[0016] The plasmid pSC101, with a plasmid copy number of about 5 per cell, is a plasmid isolated from *Salmonella* that can replicate in *E. coli* cells. The parent plasmid of pSC101 is plasmid R6-5, that is in turn derived from the plasmid R6. The origin of replication is thus the same on the plasmids pSC101, R6-5, and R6. The origin of replication of P1 contains genetic elements that provide for stable inheritance of the plasmid, making this origin of replication attractive for use as the basis of a stable 5 copy number plasmid cloning vector.

[0017] Most commonly used multi-copy cloning vectors are inherited in an unstable fashion, being lost under non-selective conditions at frequencies of between 10^{-2} to 10^{-5} per cell per generation (Summers and Sherratt, 1984). However, the multi-copy plasmid ColE1 is stably inherited, suggesting that ColE1 includes a stabilizing function. It was found that partitioning of ColE1-like plasmid between cells at division is random; this was a puzzling observation, given the stable inheritance of ColE1 versus the unstable inheritance of smaller ColE1-like cloning vectors. The puzzle was solved with the finding that plasmid multimers form with the smaller ColE1-like cloning vectors, and that this multimerization was the cause of the unstable inheritance because plasmid multimers are maintained at a lower copy number than plasmid monomers (the content of plasmid-monomer equivalents is the same per cell), and thus plasmid multimer-containing cells are more at risk of giving rise to plasmid-free segregants after cell division. A region on ColE1 was found that functioned to resolve plasmid multimers back to monomers, and was designated *cer* (for ColE1 resolution) (Summers and Sherratt, 1984).

[0018] A similar stability function was found on plasmid ColK, and the identification of a region of nucleotide sequence homology on the two plasmids allowed the *cer* site to be pinpointed to a region of about 150 bp (Summers et al., 1985). The molecular biology of the *cer* site, and the involvement of host factors in multimer resolution, has been studied extensively (Summers and Sherratt, 1988; Summers, 1989; Summers, 1991; Summers et al., 1993; Hodgman et al., 1998). At least four chromosomally-encoded proteins are involved, including ArgR, the repressor of arginine biosynthesis (Stirling et al., 1988); PepA, aminopeptidase A (Stirling et al., 1989); and the recombinases XerC (Colloms et al., 1990) and XerD (Blakely et al., 1993). This dependence on host factors is likely to put constraints on the functional host range of the *cer* system. *cer*-mediated recombination occurs in a unidirectional fashion, ensuring only the conversion of multimers to monomers (Guhathakurta and Summers, 1995; Guhathakurta et al., 1996).

[0019] Multimer resolution is necessary but not sufficient for the stable maintenance of ColE1. A promoter within *cer*, *pcer*, directs the synthesis of a 70 nucleotide untranslated RNA molecule that is not required for multimer resolution, but is essential for stable plasmid maintenance. This RNA molecule, known as RCD (regulator of cell division), arrests the division of cells containing plasmid multimers (Patient and Summers, 1993). This effect of RCD prevents multimer-containing cells from dividing and producing plasmid-free segregant cells. Transcription from *pcer* occurs almost exclusively in multimer-containing cells, with very little RCD detected in monomer-containing cells. This suggests that the *pcer* promoter is topologically constrained, requiring a degree of supercoiling only found on plasmid multimers in order for the promoter to be activated. It is not known how RCD exerts its effects on cell division. The use of the RCD molecule to create "quiescent" cells has been claimed in Summers and Rowe, United States Patent No. 6,190,867.

[0020] Thus, the present invention addresses the foregoing problems listed in the art by creating modified hybrid pBR322 or pACYC184 plasmids. Examples of the problems listed in the art are: 1) the need for either high or low copy plasmids; 2) the need for portability of the origin of replication between plasmids; and 3) the need for customizable plasmids, i.e., the ability to add specific genetic elements to a plasmid, such as the *cer* region that increases plasmid stability in culture. Some hybrid pBR322 or pACYC184 plasmids contain a *cer* site in order to provide for prolonged plasmid stability in culture. This plasmid stability is particularly desired for modified

constructs that produce a desired protein when such constructs need to be cultured for many (>20) generations in the absence of antibiotic selection.

SUMMARY OF THE INVENTION

[0021] The present invention is directed to a hybrid origin of replication comprising nucleotide sequences from an origin of replication from at least two different plasmids. The present invention includes plasmids modified to contain a portable origin of replication, preferably flanked by multiple unique restriction sites known as multiple cloning sites (MCS), creating an easily exchangeable origin of replication. Such modified plasmids will provide useful cloning tools that allow for regulation of the level of expression of desired or target gene products.

[0022] An embodiment of the present invention includes a chimeric or hybrid origin of replication comprising nucleotide sequences from an origin of replication from at least two different plasmids. Such hybrid origins of replication comprise nucleotide sequences from pBR322 (SEQ ID 1) and nucleotide sequences from ColE1 (SEQ ID 6). Another hybrid origin of replication comprise nucleotide sequences from position 1766 to AlwNI restriction site in a pBR322 plasmid linked to nucleotide sequences from an AlwNI restriction site to position 3148 in a ColE1 plasmid (SEQ ID 7). Yet another hybrid origin of replication comprises nucleotide sequences from position 1766 to AlwNI restriction site in a ColE1 plasmid linked to nucleotide sequences from an AlwNI restriction site to position 3148 in a pBr322 plasmid (SEQ ID 12). Another hybrid origin of replication comprises nucleotide sequences from pBR322 and pACYC184 (SEQ ID 28). For some embodiments of the present invention, at least 200 nucleotides, more preferably 250 nucleotides, from a one origin of replication is linked to nucleotides from a different origin of replication.

[0023] Another embodiment of the present invention includes an exchangeable origin of replication cassette comprising a nucleotide sequence of an origin of replication flanked on each side by nucleotide sequences coding for at least one cloning site; said cloning site not within a regulatory or structural coding region. In certain embodiments, the exchangeable origin of replication cassette may comprise multiple cloning sites such as: SEQ ID NO: 30 (the BglII – NsiI – NotI multiple cloning site fragment) and/or SEQ ID NO: 31 (the SacI – SpeI – BglII multiple cloning site fragment). The recognition sites for these restriction endonucleases are

AGATCT for BglII, ATGCAT for NsiI, GCGGCCGC for NotI, GAGCTC for SacI, and ACTAGT for SpeI.

[0024] Another embodiment of the present invention includes modified plasmids containing an exchangeable origin of replication cassette comprising a nucleotide sequence of an origin of replication flanked on each side by nucleotide sequences coding for at least one cloning site. Additional embodiments include a modified plasmid wherein the exchangeable origin of replication comprises the nucleotide sequences from the pBR322, ColE1, pMB1, P15A or pACYC184 origin of replication.

[0025] Yet further embodiments include a modified plasmid wherein the exchangeable origin of replication comprises a hybrid ColE1 and pMB1 origin of replication.

[0026] Embodiments of the present invention include a modified plasmid based on the pBR322 plasmid backbone, or a plasmid exhibiting at least 70% nucleotide sequence identity with the origin of replication regions of pBR322. pACYC184 and ColE1 sequences comprising the remainder of the origin of replication. This means there is an overall homology of at least 70% between the ORI of pBR322 and a second ORI being used to create the hybrid ORI. In one such embodiment the plasmid is pXT977.

[0027] This plasmid could be further modified so that multiple cloning sites flank the hybrid origin of replication. In one such embodiment, the plasmid is pXT988. An additional embodiment includes a modified plasmid based on the pBR322 plasmid backbone, or a plasmid exhibiting at least 70% nucleotide sequence identity with the origin of replication regions of pBR322 or pACYC184, comprising a pMB1 origin of replication flanked by multiple cloning sites. In one such embodiment, the plasmid is pXT995.

[0028] In additional embodiments, the modified plasmid vector further comprises bovine somatotropin (bST) expression elements, and is represented pXT757, pXT985, pXT986, pXT987, pXT996, pXT1002, pXT1003, pXT1004, pXT1007, pXT1109, or pXT1110.

[0029] Additional embodiments include a method of creating an exchangeable origin of replication cassette within a first plasmid comprising:

- [0030] (a) using primers to amplify the existing origin of replication within the first plasmid, wherein the primers comprise an annealing region and a region comprising at least one cloning site that corresponds to a cloning site in a second plasmid;

[0031] (b) isolating the PCR amplicons, said amplicons containing an origin of replication region, an annealing region and at least one cloning site.

[0032] In yet additional embodiments, the invention covers a method of exchanging an origin of replication region between a first plasmid and a second plasmid comprising:

[0033] (a) using primers to amplify the existing origin of replication within the first plasmid, wherein the primers comprise an annealing region and a region comprising at least one cloning site that corresponds to a cloning site in a second plasmid;

[0034] (b) isolating the PCR amplicons, said amplicons containing an origin of replication region, an annealing region and at least one cloning site;

[0035] (c) digesting the PCR amplicons with a restriction enzyme that recognizes the at least one cloning site;

[0036] (d) digesting the second plasmid with a restriction enzyme that recognizes the at least one cloning site so as to cut out the existing origin of replication;

[0037] (e) ligating the PCR amplicons of the first plasmid into the second plasmid.

[0038] Yet further embodiments include a bacterial host cell transformed with any of the modified plasmids described above. Additional embodiments include a kit comprising any of the modified plasmids described above. The kits may also include compatible competent host cells.

[0039] The invention may encompass a method of producing a recombinant protein of interest comprising:

[0040] (a) transforming a suitable bacterial host cell compatible with a ColE1, MB1, p15A or hybrid ColE1, MB1, p15A replication system, with any one of the modified plasmids described above containing a gene encoding a recombinant protein of interest, operatively linked to expression control nucleotide sequences; and

[0041] (b) growing a culture of said suitable bacterial host transformed with said modified plasmids under suitable conditions for expression of said recombinant protein; and

[0042] (c) recovering and purifying the protein of interest.

[0043] In yet a further embodiment, the invention may include a method of producing plasmid DNA in *E. coli* comprising:

[0044] (a) transforming a suitable *E. coli* strain with any modified plasmids described above; and

[0045] (b) growing a culture of said *E. coli* transformed with said modified plasmids under conditions which allow replication of said vector.

DESCRIPTION OF THE FIGURES

[0046] The following figures form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these figures in combination with the detailed description of specific embodiments presented herein.

[0047] **Figure 1** shows a linear map of the pBR322 origin of replication (nucleotides 1766-3148).

[0048] **Figure 2** shows the design of a modified pBR322 construct, pXT975. In panel 2A, the design of one of the primers used to create the modified plasmid is shown with a region at coordinate 3149 that is homologous to pBR322 and a long tail region that is homologous to the ColE1 origin of replication. In panel 2B, the design of a second primer is shown with a region at coordinate 2892 that is homologous to the ColE1 origin of replication and a tail region that is homologous to the pBR322 plasmid at coordinate 3149. Panel 2C, shows the modified pBR322 construct, pXT975 in which positions 2892-3148 of the origin of replication have been replaced with the corresponding region from the ColE1 origin to create a hybrid origin of replication.

[0049] **Figure 3** shows the design of a modified pBR322 construct, pXT976. In panel 3A, the design of one of the primers used to create the modified plasmid is shown with a region at coordinate 1667 that is homologous to pBR322 and a long tail region that is homologous to the ColE1 origin of replication. In panel 3B, the design of a second primer is shown with a region at coordinate 2892 that is homologous to the ColE1 origin of replication and a tail region that is homologous to the pBR322 plasmid at coordinate 1667. Panel 3C, shows the modified pBR322 construct, pXT976 in which positions 1766-2892 of the origin of replication have been replaced with the corresponding region from the ColE1 origin to create a hybrid origin of replication.

[0050] **Figure 4** shows a comparison of the origin of replication regions among pBR322, pXT975, pXT976, and pXT977. The modified pBR322 construct, pXT977, has the entire

pBR322 (pMB1) origin of replication replaced with the origin of replication of ColE1 (nucleotides 1766-3148).

[0051] **Figure 5** shows the construction of pXT988, a modified pBR322 construct with the ColE1 origin of replication region flanked by multiple cloning sites (MCS). In panel 5A, the design of one of the primers used to create the modified plasmid is shown with a region at coordinate 3430 that is homologous to pBR322 and a long tail region containing sequences of multiple restriction enzyme recognition sites and a region of sequences that is homologous to the ColE1 origin of replication. In panel 5B, the design of a second primer is shown with a region at coordinate 2892 that is homologous to the ColE1 origin of replication and a tail region containing sequences of multiple restriction enzyme recognition sites and a region of sequences that is homologous to the pBR322 plasmid at coordinate 3430. In panel 5C, the design of a second primer is shown with a region at coordinate 1667 that is homologous to the pBR322 origin of replication and a tail region containing sequences of multiple restriction enzyme recognition sites and a region of sequences that is homologous to the ColE1 plasmid. In panel 5D, the design of a second primer is shown with a region at coordinate 2892 that is homologous to the ColE1 origin of replication and a tail region containing sequences of multiple restriction enzyme recognition sites and a region of sequences that is homologous to the pBR322 plasmid at coordinate 1667. Panel 5E shows the modified pBR322 construct, pXT988, which contains the entire ColE1 origin of replication, flanked by MCS.

[0052] **Figure 6** shows features of plasmids with the origin of replication regions flanked by multiple cloning sites (MCS). Shown in panel 6A is the plasmids pXT995, where the origin of replication is entirely from pBR322 and is flanked by MCS. Panel 6B shows hybrid plasmid pXT1001, where the origin of replication contains segments from both the ColE1 and the pBR322 (pMB1) origins of replications and is flanked by MCS. Panel 6C shows hybrid plasmid pXT1000, where the origin of replication contains segments from both the ColE1 and the pBR322 (pMB1) origins of replications and is flanked by MCS. Panel 6D shows hybrid plasmid pXT988, where the origin of replication is entirely from ColE1, and is flanked by MCS.

[0053] Figure 7 shows the features of pXT1092, in which the pBR322 (pMB1) origin of replication flanked by multiple cloning sites (MCS) has been flipped into the opposite orientation of that found on pXT995.

[0054] Figure 8 shows a linear map of the origin of replication region on the plasmid pACYC184 (nucleotides 580-1407) and the single restriction enzyme site at coordinate 596 for the restriction enzyme BstZ171.

[0055] Figure 9 shows a comparison of the nucleotide sequences of the origins of replication from pBR322 (top nucleotide sequence, SEQ ID NO:1) and pACYC184 (lower nucleotide sequence, SEQ ID NO:24).

[0056] Figure 10 shows the nucleotide sequence of the NsiI-NotI fragment of the hybrid plasmid pXT1007 (SEQ ID NO:32) containing a *cer* site.

[0057] Figure 11 shows a linear map of the origin of replication region of plasmid pXT1221, containing a *cer* site.

DETAILED DESCRIPTION OF THE INVENTION

[0058] The following definitions are provided in order to aid those skilled in the art in understanding the detailed description of the present invention.

[0059] The terms "expression construct" and "recombinant expression construct" and "expression system" will be understood to describe genetically-engineered nucleic acid sequences encoding at a minimum an origin of replication, a selectable marker and a gene or polypeptide-encoding nucleic acid of interest to be expressed in a recipient host cell.

[0060] The term "plasmid" or "vector" will be understood to include any extrachromosomal covalently continuous double-stranded nucleic acid molecule.

[0061] The term "copy number" is the number of molecules of a particular type of plasmid on or in a cell or part of a cell. This will be understood to describe a characteristic of a recombinant expression construct present in a host cell in greater than a single copy per cell. Most plasmids are classified by the terms "multiple copy number," "low copy number" or "high copy number," which describes the ratio of plasmid/chromosome molecules.

[0062] The term "regulatable promoter" is intended to encompass DNA sequences that mediate transcription of a nucleic acid in a cell. Regulatable promoters are distinguished from promoters that are not regulatable in that regulatable promoters are operatively linked to "cis-acting transcription control elements" that will be understood to be nucleic acid sequences that regulate

or control transcription of a polypeptide-encoding nucleic acid. As used herein, the term "cis-acting transcription control element" is particularly directed to nucleic acid sequences that make said regulatable promoter "inducible," as that term is defined herein below. Said regulatable promoters of the invention comprising said cis-acting transcription control elements are operatively-linked to polypeptide-encoding nucleic acids and control transcription thereof in a cell, most preferably a bacterial cell, and more preferably an *E. coli* cell, into which a recombinant expression construct of the invention has been introduced. Most preferably, the transcription control of the regulatable promoters of the invention is mediated by interaction between the cis-acting transcription control elements with the trans-acting transcription factors encoded by the recombinant expression constructs of the invention. Regulatable promoters such as the bacteriophage lambda P_L promoter, the promoters of the *lac* operon, the *trp* operon, and the *ara* operon, as well as some of their derivatives, have been widely used to control gene expression. Another family of regulatable promoters is the synthetic cpex promoter series (described in Bogosian *et al.*, United States Patent 6,617,130), which is induced by nalidixic acid.

[0063] The term "operatively linked" is intended to describe the linkage between nucleic acids wherein the position and proximity of the linkage ensures coupled replication and is sufficient and appropriate to be recognized by trans-acting transcription factors and other cellular factors whereby polypeptide-encoding nucleic acid is efficiently expressed under appropriate conditions.

[0064] The term "origin of replication" or "ORI" as used herein is intended to encompass regions of nucleotides that are necessary for replication of a plasmid. Some of examples of origins of replication: nucleotides 1766-3148 of pBR322; nucleotides 1667-2892 of ColE1; and nucleotides 580-1407 of pACYC184.

[0065] The terms "hybrid" or "chimeric" will be understood to mean any plasmid containing nucleotide sequences from two or more plasmids.

[0066] The terms "hybrid origin of replication" or "chimeric origin of replication" as used herein are intended to encompass nucleotide sequences from a first plasmid's origin of replication combined or linked with nucleotide sequences from a second plasmid's origin of replication in order to create a region of nucleotides that allows for the replication of a plasmid. Some

examples of plasmids containing hybrid origin of replications are: pXT975 and pXT976.

[0067] The term "restriction enzyme" will be understood to mean any of a group of enzymes, produced by bacteria, which cleave molecules of DNA internally at specific base sequences. Examples of restriction enzymes would include: BspEI; BglII; NsiI; NotI; SacI; SpeI; and AlwNI

[0068] The term "restriction site" will be understood to mean a sequence of bases in a DNA molecule that is recognized by a restriction enzyme.

[0069] The term "multiple cloning sites" will be understood to mean a region of nucleotides containing more than one restriction site. Examples of nucleotides sequences containing multiple cloning sites would include: SEQ ID 14; SEQ ID 15; SEQ ID 19; and SEQ ID 20.

[0070] The term "PCR amplicon" will be understood to mean amplification products of a polymerase chain reaction (PCR).

[0071] For the purposes of this invention, with regard to polypeptide expression, the terms "elevated" or "elevated expression" or "over expression" are intended to indicate that the amount of the polypeptide produced in a cell, preferably a bacterial cell and more preferably an *E. coli* cell transformed with at least one of the recombinant expression constructs of the invention, is higher, more preferably much higher, than the amount of the polypeptide produced either natively or using other recombinant expression constructs. For endogenously produced polypeptides, the term is intended to mean increased expression compared with endogenous expression levels. For heterologous polypeptides, the term is intended to reflect increased production of said heterologous polypeptides associated with conventional recombinant or genetic engineering-related expression vectors, systems and methods.

[0072] The present invention provides nucleic acids, recombinant expression constructs, bacterial cells, reagents and methods for regulating bacterial gene expression, for over expression of desired or target polypeptides in bacteria.

[0073] Hybrid origins of replication comprising nucleotide sequences from an origin of replication from at least two different plasmids can be created. Sequences from pBR322, pACYC184, p15A, MB1 or ColE1 can be combined to create a hybrid origin of replication. Plasmids can also be modified to contain a portable hybrid origin of replication. Depending on the needs of the user, different regions of different origin of replications could be used in order to

tailor the characteristics of the origin of replication to suit the user's needs. For some embodiments of the present invention, at least 200 nucleotides, more preferably 250 nucleotides, from a one origin of replication is linked to nucleotides from a different origin of replication.

[0074] Plasmids can also be modified to contain a portable hybrid origin of replication. A hybrid origin of replication is created that is flanked by multiple unique restriction sites known as multiple cloning sites (MCS). With the MCS on each side of the origin of replication, the hybrid origin of replication is easily exchangeable or portable. Such modified plasmids will provide useful cloning tools that allow for regulation of the level of expression of desired or target gene products because the copy number of a plasmid could be controlled.

[0075] The present invention encompasses a number of modified plasmids that were created by modifying the pBR322 plasmid. One of these modified pBR322 plasmids, the pXT995 plasmid, has the standard pMB1 origin of replication region normally present on pBR322, flanked by multiple unique restriction sites (a multiple cloning site, or MCS). Another modified pBR322 plasmid of the present invention, pXT988, has had the pMB1 origin of replication replaced with the ColE1 origin. It also has the desirable feature of having the origin region flanked by MCS. The pXT995 and pXT988 plasmids of the present invention allow for easy replacement of the origin of replication with nearly any desired origin of replication. These plasmids also retain the desirable features of the parent pBR322 plasmid.

[0076] The pXT995 and pXT988 plasmids will be useful in situations where it is desirable to modify the plasmid copy number, by exchanging the origin of replication such as when a low copy number is desirable, for over-expressing a gene that is not well-tolerated by the host cell. In other circumstances, however, it may be desirable to have a high copy number plasmid, such as when purifying an over-expressed protein. Additionally, it may be desirable to have a differentially regulatable origin of replication. All of these options or adaptations are available by using the modified plasmids pXT995 or pXT988 of the present invention or creating other customized plasmids based on the disclosure of the present invention.

[0077] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the Inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its

practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the scope of the invention.

EXAMPLES

[0078] **Culture Procedures.** Bacteria were routinely cultured in Luria-Bertani (LB) medium or LB agar supplemented as appropriate with ampicillin (100 µg/ml), chloramphenicol (25 µg/ml), tetracycline (10 µg/ml), kanamycin (25 µg/ml), streptomycin (25 µg/ml), or spectinomycin (25 µg/ml).

[0079] **DNA procedures.** Plasmid DNA was isolated using an alkaline lysis method and purified if necessary using a Qiaprep Spin Miniprep (Qiagen). Polymerase chain reaction (PCR) was done in a Roche GeneAmp PCR System 2700, using the Roche PCR Master Kit. Custom primers were synthesized by InVitrogen. Restriction digestions were carried out according to the enzyme manufacturers' instructions for each restriction endonuclease. For isolation of DNA fragments, the fragments were separated on 0.9% (wt/vol) agarose gels and isolated using a Qiaquick Gel Extraction Kit (Qiagen). Roche T4 DNA Ligase was used for DNA ligations. Plasmid transformations were carried out in a BioRad Micropulser, using InVitrogen ElectroMax DH5α competent cells. For nucleotide sequencing, automated sequencing was carried out using an ABI Prism 3730XL DNA sequencer (PE Biosystems) and Big Dye terminator mixes.

[0080] The present invention involves genetically modifying the origin of replication region of pBR322 (pMB1) from coordinates 1766-3148 and to create an exchangeable origin of replication region flanked by unique restriction sites (e.g. multiple cloning sites, MCS). The technique of "splicing by overlap extension" (SOE) was utilized to create the modified pBR322 constructs of the present invention; however, other suitable methods could also be utilized. These SOE methods are well known and are described by Horton *et al.* in Gene, 77: 61-68 (1989).

[0081] The plasmid pBR322 has a copy number of about 20-30, a number determined in part by its pMB1 origin of replication. An aspect of the present invention involves replacing the pMB1 origin of replication region of pBR322 with a number of different origin constructs, including creating several hybrid plasmids such as pXT975, pXT976, pXT1000, and pXT1001, which have a modified origin of replication region. These plasmids contain a hybrid origin of replication

comprising of pMB1 origin of replication nucleotide sequences and ColE1 origin of replication nucleotide sequences.

[0082] Since ColE1 has been one of the standard plasmids utilized in many of the traditional experiments on plasmid origins of replication, the construction of a modified pBR322-based plasmid that has its origin of replication region replaced with the ColE1 origin of replication region provides a very useful tool for cloning and expression constructs, as well as for future origin of replication experiments. Thus, another aspect of the present invention involves creating a modified pBR322 construct in which the entire origin of replication has been replaced with a corresponding origin from ColE1, and is also flanked by multiple cloning sites (MCS).

[0083] When an origin of replication is flanked by MCS, it results in an origin of replication region that is easily exchangeable with other desired origins of replication. An example of this type of modified construct includes plasmid pXT988. The plasmid pXT988 serves as a template from which to create any desired, pBR322-based plasmid construct having any desired origin of replication, since it has an easily replaceable origin cassette flanked by multiple cloning sites. A related plasmid of the present invention, pXT995, has the unmodified pBR322 (pMB1) origin of replication flanked by MCS, also facilitating the replacement of the origin with any origin of replication that has desired features such as a lower copy number, a higher copy number, or other desirable feature.

[0084] In the design of these various plasmid modifications, the pMB1 origin of replication region on pBR322 was replaced in stages, with varying amounts of the ColE1 origin of replication region. A map showing the unique restriction sites on pBR322 is shown in Figure 1. Two MCS's, the BglII – NsiI - NotI fragment with the **SEQ ID NO: 30**; AGATCTATGCATGCGGCCGC; and the SacI – SpeI - BglII fragment with **SEQ ID NO: 31**; GAGCTCACTAGTAGATCT were used for constructing some of the desired modified plasmids.

[0085] The initial splicing by overlap extension (SOE) manipulations use two existing unique restriction sites on pBR322 that are located outside of the 1766-3148 origin of replication region. At this stage of the plasmid manipulations it did not matter that these sites were some distance outside the origin of replication region. The restriction sites used were BspEI at coordinate 1667, and BsaI at coordinate 3430. A third site was used to facilitate the SOE work, the unique

AlwNI site within the origin of replication region at coordinate 2892. This AlwNI site is also present at the same relative position within the ColE1 origin of replication.

Example 1. Construction of pXT975.

[0086] Primers were used to amplify by PCR the region on pBR322 from about coordinate 3149 within the origin of replication region to about coordinate 3500, past the BsaI site at coordinate 3430, as shown in Figure 2. The nucleotide sequence of pBR322 is described in **SEQ ID NO: 1**. The nucleotide sequence given here is different from that found in Genbank, as more recent nucleotide sequencing of the plasmid pBR322 by the inventors has shown differences with the Genbank nucleotide sequence; the nucleotide sequence given here is believed by the inventors to be the correct nucleotide sequence of pBR322.

[0087] The following primers were utilized for these initial amplification steps:

SEQ ID NO:2: 5'-GCTCGGCCCTTCCGGCTGGC-3'

SEQ ID NO:3:

5'-TTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTAATCTTTTCTAC
GGGGTCTGACGCTCAGTGGAAACGAAAACCTCACGTTAAGGGATTTTGGTC-3'

The primer for the region at coordinate 3149 (SEQ ID NO:3) included a long tail matching the corresponding region of the ColE1 origin of replication.

[0088] The resulting 399 base pair PCR fragment was used as the source of a 399 base pair primer (**SEQ ID NO:4**),

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1  GCTCGGCCCT TCCGGCTGGC TGGTTTATTG CTGATAAATC TGGAGCCGGT
51  GAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCC
101 CTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATG
151 AACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGG
201 TAACTGTCAG ACCAAGTTTA CTCATATATA CTTTAGATTG ATTTAAAACT
251 TCATTTTAA TTTAAAAGGA TCTAGGTGAA GATCCTTTTT GATAATCTCA
301 TGACCAAAAT CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC
351 GTAGAAAAGA TTAAAGGATC TTCTTGAGAT CCTTTTTTTC TGCGCGTAA

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[0089] along with a second, regular primer matching the plasmid ColE1 origin of replication just upstream of the AlwNI site (**SEQ ID NO:5** 5'- CCTTCTAGTGTAGCCGTAGTCGGGCC-3'), to generate a PCR fragment from ColE1 plasmid DNA.

[0090] The nucleotide sequence of plasmid ColE1 is described in **SEQ ID NO:6**. The nucleotide sequence given here is different from that found in Genbank, as more recent nucleotide sequencing of the plasmid ColE1 by the inventors has shown differences with the Genbank nucleotide sequence; the nucleotide sequence given here is believed by the inventors to be the correct nucleotide sequence of ColE1. The resulting PCR fragment was digested with the restriction endonucleases AlwNI and BsaI to yield a fragment that was inserted into pBR322, yielding plasmid pXT975, which has the region from the AlwNI site at coordinate 2892 to the end of the pBR322 origin of replication region at coordinate 3148, replaced by the corresponding region of the ColE1 origin of replication. The nucleotide sequence of pXT975 is described in **SEQ ID NO:7**.

Example 2. Construction of pXT976.

[0091] Primers were used to amplify by PCR the region on pBR322 from just before the BspEI site at coordinate 1667 to coordinate 1766 as shown in Figure 3. The following primers were utilized for these initial amplification steps:

(**SEQ ID NO:8** 5'-GCGACCTGAGCAACAACATGAATGG-3')

(**SEQ ID NO:9**

5'-TTACTTGAACGCTGTGAGGGTAAACAACCTGGCGGTATGGATGCGGCGGGA
CCAGAGAAAAATCACTCAGGGTCAATGCCAGCGCTTCGTTAATACAGATG-3')

The primer for the region at coordinate 1765 (**SEQ ID NO:9**) included a long tail matching the corresponding region of the ColE1 origin of replication.

[0092] The resulting 236 base pair PCR fragment was used as the source of a 236 base pair primer (**SEQ ID NO:10**),

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1  GCGACCTGAGCAACAACATGAATGGTCTTCGGTTTCCGTG TTTCGTAAAG
51 TCTGGAAACGCGGAAGTCAGCGCCCTGCACCATTATGTTCCGGATCTGCA
101 TCGCAGGATGCTGCTGGCTACCCTGTGGAACACCTACATCTGTATTAACG
151 AAGCGCTGGCATTGACCCTGAGTGATTTTTCTCTGGTCCCGCCGCATCCA
201 TACCGCCAGTTGTTTACCCTCACAGCGTTCAAGTAA

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along with a second, regular primer matching the ColE1 origin of replication just past the AlwNI site (**SEQ ID NO:11** 5'-GCCCCGACCGCTGCGCCTTATCCGG-3'), to generate a PCR fragment from ColE1 plasmid DNA.

[0093] The resulting PCR fragment was digested with BspEI and AlwNI and inserted into pBR322 as shown in Figure 3C. This yielded plasmid pXT976, which has the region from the end of the pBR322 origin of replication at coordinate 1766 to the AlwNI site at coordinate 2892 replaced by the corresponding region from the ColE1 origin of replication. The nucleotide sequence corresponding to pXT976 is described in **SEQ ID NO: 12**.

Example 3. Construction of pXT977.

[0094] The final two PCR fragments as described above for the construction of pXT975 and pXT976, were cut with AlwNI, ligated together, and then amplified with PCR primers matching the tail regions (**SEQ ID NO:2**, and **SEQ ID NO:8**, shown above) outside the BspEI and BsaI sites.

[0095] The resulting PCR fragment was digested with BspEI and BsaI and inserted into pBR322. This yielded plasmid pXT977, which has the pBR322 origin of replication region from coordinates 1766 to 3148 replaced with the corresponding region from the ColE1 origin of replication. The nucleotide sequence of pXT977 is described as **SEQ ID NO:13**.

Example 4. Comparative maps of the origin of replication regions of pBR322, pXT975, pXT976, and pXT977.

[0096] The construction of the three plasmids described in Examples 1-3 yielded modified pBR322-based plasmids containing the following homogenous (one source) or hybrid/chimeric origin of replication regions as shown in Figure 4:

pBR322: origin of replication region from 1766-3148 from pMB1 (homogeneous origin of replication);

pXT975: origin of replication region from 1766 to AlwNI from pMB1, and from AlwNI to 3148 from ColE1 (hybrid origin of replication);

pXT976: origin of replication region from 1766 to AlwNI from ColE1, and from AlwNI to 3148 from pMB1 (hybrid origin of replication);

pXT977: origin of replication region from 1766-3148 from ColE1 (homogeneous origin of replication).

Example 5. Experimentally determined plasmid copy numbers for pBR322, and modified pBR322-based plasmids pXT975, pXT976, and pXT977.

[0097] The copy numbers for plasmids pBR322, pXT975, pXT976, and pXT977 were determined in strain LBB427, which is the standard wild-type *E. coli* K-12 strain W3110 with a mutation inactivating the *fhuA* gene. The copy number measurements were made on two sets of shake flask cultures grown in L-broth plus ampicillin (100 micrograms per milliliter). At each copy number measurement time point, a sample of the culture was diluted and plated on L-broth agar plates containing ampicillin (100 micrograms per milliliter) in order to get a count of plasmid-containing cells per milliliter of culture. From this same sample of the culture, plasmid DNA was isolated and quantified. Plasmid DNA quantification was performed by linearization of the plasmid with a restriction enzyme that cut the plasmid molecule at only one site, running the linearized plasmid DNA on a polyacrylamide gel along, staining the gel with ethidium bromide, and performing scanning densitometry on the stained plasmid DNA band. The resultant densitometric scan reading was compared to the densitometric scan readings obtained from known amounts of a plasmid DNA standard to determine the amount of plasmid DNA per milliliter of culture. This amount was converted to molecules of plasmid DNA per milliliter of culture, and then divided by the number of cells per milliliter of culture to yield the plasmid copy number in terms of molecules of plasmid DNA per cell. Table 1 shows the results of this experiment; at each time point, two plasmid copy numbers are given, showing the results from the duplicate cultures that were assayed.

Table 1

Plasmid copy number during growth in shake flasks containing L-broth medium plus ampicillin and tetracycline

Plasmid	(hours after inoculation)
	<u>7.5 hrs</u>
pBR322	32, 34
pXT975	8, 8
pXT976	17, 23
pXT977	6, 9

[0098] The above results indicate that plasmid copy number, when compared to that of pBR322 with its pMB1 origin (and also to pXT976 that has a complete pMB1 origin), is reduced when the origin of replication region from coordinate 2892 (the AlwNI site) to position 3148 is replaced with the corresponding region from ColE1 (as on plasmids pXT975 and pXT977). These results illustrate the utility of the present invention in providing new plasmid vectors with two ranges of plasmid copy numbers, namely plasmids pXT975 and pXT977 with plasmid copy numbers of about 10, and plasmid pXT976 with a plasmid copy number of about 20.

Example 6. Construction of pXT988, a modified pBR322 plasmid containing the ColE1 origin of replication region flanked by multiple cloning sites creating a replaceable origin of replication region.

[0099] To construct a plasmid with the origin of replication region flanked by multiple cloning sites, the SOE constructions using the initial primers containing the long ColE1 tails described in Examples 1-4 were repeated, but these primers were changed slightly to include multiple cloning sites at the junction between pBR322 and ColE1 nucleotide sequences as shown in Figure 5. The primer (SEQ ID NO:14):

5'-GCTGTGAGGGTAAACAACTGGCGGTATGGATGCGGCGGGGCGGCCGCATG
CATAGATCTACCAGAGAAAAATCACTCAGGGTCAATGCCAGCGCTTCGTT-3'

was substituted for the primer SEQ ID NO:9, and the primer (SEQ ID NO:15):

5'-AGAAAAAAGGATCTCAAGAAGATCCTTTAATCTTTTCTACGAGCTCACT
AGTAGATCTGGGGTCTGACGCTCAGTGGAACGAAAACACGTTAAGGGA-3'

was substituted for the primer SEQ ID NO:3.

The nucleotide sequence of the multiple cloning site (MCS) included on the primer SEQ ID NO:14 included recognition sites for the restriction endonucleases BglII (AGATCT), NsiI (ATGCAT), and NotI (GCGGCCGC).

The nucleotide sequence of the multiple cloning site (MCS) included on the primer SEQ ID NO:15 included recognition sites for the restriction endonucleases SacI (GAGCTC), SpeI (ACTAGT), and BglII (AGATCT).

[0100] Preparation of the BsaI-AlwNI fragment with a multiple cloning site is shown in Figures 5A and 5B. The primers SEQ ID NO:2 and SEQ ID NO:15 were used to generate a 409 base

pair PCR fragment. This PCR fragment was used as the source of a 409 base pair primer (SEQ ID NO:16),

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1   GCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGT
51  GAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCC
101 CTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATG
151 AACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGG
201 TAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAACT
251 TCATTTTAAATTTAAAAGGATCTAGGTGAAGATCCTTTTGTATAATCTCA
301 TGACCAAATCCCTTAACGTGAGTTTTCTGTTCCACTGAGCGTCAGACCCC
351 AGATCTACTAGTGAGCTCGTAGAAAAGATTAAAGGATCTTCTTGAGATCC
401 TTTTTTCT

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that was used along with a second, regular primer matching the ColE1 origin of replication just upstream of the AlwNI site (SEQ ID NO:5), to generate a PCR fragment from ColE1 plasmid DNA.

[00101] Preparation of the AlwNI-BspEI fragment with a multiple cloning site is shown in Figure 5C and 5D. The primers SEQ ID NO:8 and SEQ ID NO:14 were used to generate a 246 base pair PCR fragment. This PCR fragment was used as the source of a 246 base pair primer (SEQ ID NO:17),

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1   GCGACCTGAGCAACAACATGAATGGTCTTCGGTTTCCGTGTTTCGTAAAG
51  TCTGGAAACGCGGAAGTCAGCGCCCTGCACCATTATGTTCCGGATCTGCA
101 TCGCAGGATGCTGCTGGCTACCCTGTGGAACACCTACATCTGTATTAACG
151 AAGCGCTGGCATTGACCCTGAGTGATTTTTCTCTGGTAGATCTATGCATG
201 CGGCCGCCCCGCGCATCCATACCGCCAGTTGTTTACCCTCACAGC

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that was used along with a second, regular primer matching the ColE1 origin of replication just past the AlwNI site (SEQ ID NO:11), to generate a PCR fragment from ColE1 plasmid DNA.

[00102] The final two PCR fragments made as described above were cut with AlwNI, ligated together, and then amplified with PCR primers matching the tail regions outside the BspEI and

BsaI sites (SEQ ID NO:2 and SEQ ID NO:8). The resulting PCR fragment was digested with BspEI and BsaI and inserted into pBR322.

[00103] The resulting plasmid is pXT988, as shown in Figure 5E, has the pBR322 (pMB1) origin of replication region from coordinates 1766 to 3148 replaced by the corresponding region from the ColE1 origin of replication, and also has the ColE1 origin of replication region flanked by multiple cloning sites, creating a replaceable ColE1 origin of replication region. The restriction sites in the two multiple cloning sites are also shown in the Figure 5E. The nucleotide sequence of pXT988 is described as **SEQ ID NO:18**.

Example 7. Construction of pXT995, a modified pBR322 plasmid with the pMB1 origin of replication region flanked by multiple cloning sites.

[00104] The construction of plasmid pXT988, which is a modified pBR322-based plasmid with the pMB1 origin of replication region replaced by the ColE1 origin of replication region, and flanked by multiple cloning sites, provides for the quick and easy replacement of this origin of replication with any desired alternative origin of replication. Since pXT988 has the ColE1 origin of replication region, the obvious first choice in demonstrating the utility of this new tool was to replace the ColE1 origin of replication region with the pBR322 (pMB1) origin of replication region.

[00105] The first step to exchange the pMB1 origin of replication for the ColE1 origin of replication was to use primers located just inside where the multiple cloning sites were located, to amplify the pMB1 origin of replication region of pBR322. The primers included tails with the same multiple cloning sites, and are given as **SEQ ID NO:19**

[00106] 5'-AGGAAGATCTATGCATGCGGCCGCCCCGCGCATCCATACCGCCAGTTG-3'
and **SEQ ID NO:20**

5'-AGGAAGATCTACTAGTGAGCTCGTAGAAAAGATCAAAGGATCTTCTTG-3'

[00107] The nucleotide sequence of the multiple cloning site (MCS) included on the primer SEQ ID NO:19 included recognition sites for the restriction endonucleases BglII (AGATCT), NsiI (ATGCAT), and NotI (GCGGCCGC). The nucleotide sequence of the multiple cloning site (MCS) included on the primer SEQ ID NO:20 included with recognition sites for the restriction endonucleases SacI (GAGCTC), SpeI (ACTAGT), and BglII (AGATCT).

[00108] The resulting PCR fragment was digested with NotI and SpeI, and used to replace the ColE1 origin of replication region on pXT988 to yield the plasmid pXT995, as shown in Figure 6A. Plasmid pXT995 has the pBR322 (pMB1) origin of replication region and also has the flanking multiple cloning sites, creating a pMB1 origin cassette. The nucleotide sequence for plasmid pXT995 is described in **SEQ ID NO:21**.

Example 8. Construction of pXT1000, a modified pBR322 plasmid with a hybrid origin of replication flanked by MCS.

[00109] The plasmid pXT1000 was constructed by inserting the AlwNI-SpeI fragment from pXT996 (this fragment carries the origin of replication region of pBR322 from the AlwNI site to the MCS) into pXT988. This yielded a plasmid with a composite or hybrid origin of replication, with the region of the origin of replication from the MCS to the AlwNI site being from the ColE1 ORI, and the region of the origin of replication from the AlwNI site to the MCS being from the pMB1 ORI. The resulting plasmid is shown in Figure 6C. The nucleotide sequence for plasmid pXT1000 is described in **SEQ ID NO:22**.

Example 9. Construction of pXT1001, with a composite origin of replication flanked by MCS; ColE1 from AlwNI to the MCS.

[00110] The plasmid pXT1001 was constructed by inserting the NsiI-AlwNI fragment from pXT996 (this fragment is the pMB1 origin of replication region from the MCS to the AlwNI site) into pXT988. This yielded a plasmid with a composite or hybrid origin of replication, with the region of the origin of replication from the MCS to the AlwNI site being from the pMB1 ori, and the region of the origin of replication from the AlwNI site to the MCS being from the ColE1 ORI. The hybrid plasmid pXT1001 is shown in Figure 6B and pXT988 is shown in Figure 6D. The nucleotide sequence of plasmid pXT1001 is given as **SEQ ID NO:23**.

[00111] The construction of the pXT995, pXT1001, pXT1000, and pXT988 plasmids described above yielded plasmids containing the following homogeneous or hybrid/composite origin of replications, all flanked by MCS:

pXT995: origin of replication region from 1766-3148 from pBR322, flanked by MCS (homogeneous origin of replication);

pXT1001: origin of replication region from 1766 to AlwNI from pBR322, and from AlwNI to 3148 from ColE1, flanked by MCS (hybrid/chimeric origin of replication);

pXT1000: origin of replication region from 1766 to AlwNI from ColE1, and from AlwNI to 3148 from pBR322, flanked by MCS (hybrid/chimeric origin of replication);

pXT988: origin of replication region from 1766-3148 from ColE1, flanked by MCS (homogeneous origin of replication).

Table 3 details the plasmid constructs with novel origin of replications.

TABLE 3

Plasmid constructs with novel origins of replication

Cloning Vector	Origin Of Replication (ORI)	Plasmid SEQ ID No.
pBR322	origin of replication region (positions 1766-3148) taken from pBR322 (pMB1)	1
pXT995	origin of replication region (positions 1766-3148) taken from pBR322 (pMB1), flanked by MCS	21
pXT975	hybrid origin of replication region with one part of the region (position 1766 to AlwNI restriction site) taken from pBR322, and the other part of the region (from AlwNI restriction site to position 3148) taken from ColE1	7
pXT1001	hybrid origin of replication region with one part of the region (position 1766 to AlwNI restriction site) taken from pBR322, and the other part of the region (from AlwNI restriction site to position 3148) taken from ColE1, flanked by MCS	23
pXT976	hybrid origin of replication region with one part of the region (position 1766 to AlwNI restriction site) taken from ColE1, and the other part of the region (from AlwNI restriction site to position 3148) taken from pBR322	12
pXT1000	hybrid origin of replication region with one part of the region (position 1766 to AlwNI) from ColE1, and the other part of the region (from AlwNI to 3148) from pBR322, flanked by MCS	22
pXT977	origin of replication region (positions 1766-3148) from ColE1	13
pXT988	origin of replication region (positions 1766-3148) from ColE1, flanked by MCS	18
pXT1092	inverted origin of replication region (positions 1766-3148) taken from pBR322 (pMB1), flanked by MCS (positions 1766-3148 are inverted compared to SEQ ID NO. 1)	33
pXT1091	pACYC184 origin of replication region (BstZ17I-SacI restriction fragment from pXT1094) inserted into pXT995	28
pXT1094	pACYC184 origin of replication region (position 517-1464) inserted into MCS of pXT995	27
pXT1109	pACYC184 origin of replication region plus pBR322 <i>rop</i> gene sequence	34

Example 10. Plasmid copy numbers of plasmids with homogeneous pMB1 or hybrid pMB1/ColE1 origin of replication regions, with or without flanking MCS.

[00112] The copy numbers were determined in strain LBB427, which is the standard wild-type *E. coli* K-12 strain W3110 with a mutation inactivating the *fhuA* gene. The copy number measurements were made on two sets of shake flask cultures grown in L-broth plus ampicillin (100 micrograms per milliliter). The copy number measurements were made as described above. Table 4 shows the results of this experiment; at each time point, two plasmid copy numbers are given, showing the results from the duplicate cultures that were assayed.

TABLE 4

Plasmid copy number during mid-log phase of growth in shake flasks containing L-broth medium plus ampicillin

Plasmid	<u>7.5 hours</u> (hours after inoculation)
pBR322	32, 34
pXT995	30, 34
pXT975	8, 8
pXT1001	8, 12
pXT976	17, 23
pXT1000	24, 24
pXT977	6, 9
pXT988	11, 13

[00113] The results from these experiments show that creating exchangeable origin of replication cassettes in modified pBR322 plasmids (i.e., the origin of replication region was flanked with MCS) did not alter the copy numbers of the plasmids, when compared with plasmids lacking the MCS. Also, a previously observed trend was observed again: when the origin of replication region from the AlwNI to 3148 is from ColE1 (as on plasmids pXT975, pXT1001, pXT977, and pXT988), the copy number is reduced versus when that same origin of replication region is from pBR322 (as on the plasmids pBR322, pXT995, pXT976, and pXT1000). These results illustrate the utility of the present invention in providing new plasmid vectors with two ranges of plasmid copy numbers, namely plasmids pXT975, pXT977, pXT988, and pXT1001 with plasmid copy numbers of about 10, and plasmids pXT976, pXT995, and pXT1000 with plasmid copy numbers of about 20.

Example 11. Construction of bovine somatotropin (bST) expression plasmids with either homogeneous pMB1, ColE1, or hybrid pMB1/ColE1 origins of replication.

[00114] Bovine somatotropin (bST) is a natural protein produced in the pituitary glands of all cattle and it helps adult cows produce milk. Known in the art are several plasmids that contain the genetic elements necessary for bST protein expression. The bST expression region from one such plasmid, pXT757 (identical to pXT709, described in Bogosian *et al.*, United States Patent 6,828,124), was moved into each one of the modified pBR322-based plasmids: pXT975; pXT976, pXT977, pXT988, pXT995, pXT1000, and pXT1001. The plasmid pXT757 contains the synthetic cpex-20 promoter (described in Bogosian *et al.*, United States Patent 6,617,130), driving the expression of a synthetic bovine somatotropin (bST) gene. The cpex-20 promoter was designed to be regulated by the LexA repressor protein of the *E. coli* SOS regulon, and as such is inducible by the addition of malidixic acid. The EcoRI-BamHI fragment of pXT757 (containing the complete bST expression region from pXT757) was inserted into each of plasmids pXT975, pXT976, and pXT977. A similar EcoRI-SalI fragment of pXT757 was inserted into each of plasmids pXT988, pXT995, pXT1000, and pXT1001. These constructions yielded the bST expression plasmids shown in Table 5 below.

TABLE 5

bST expression plasmids

Bst Expression Vector	Cloning Vector	Origin Of Replication (ORI)
pXT757	pBR322	origin of replication region (positions 1766-3148) taken from pBR322 (pMB1)
pXT996	pXT995	origin of replication region (positions 1766-3148) taken from pBR322 (pMB1), flanked by MCS
pXT985	pXT975	hybrid origin of replication region with one part of the region (position 1766 to AlwNI restriction site) taken from pBR322, and the other part of the region (from AlwNI restriction site to position 3148) taken from ColE1
pXT1003	pXT1001	hybrid origin of replication region with one part of the region (position 1766 to AlwNI restriction site) taken from pBR322, and the other part of the region (from AlwNI restriction site to position 3148) taken from ColE1, flanked by MCS
pXT986	pXT976	hybrid origin of replication region with one part of the region (position 1766 to AlwNI restriction site) taken from ColE1, and the other part of the region (from AlwNI restriction site to position 3148) taken from

pXT1002	pXT1000	pBR322 hybrid origin of replication region with one part of the region (position 1766 to AlwNI) from ColE1, and the other part of the region (from AlwNI to 3148) from pBR322, flanked by MCS
pXT987	pXT977	origin of replication region (positions 1766-3148) from ColE1
pXT1004	pXT988	origin of replication region (positions 1766-3148) from ColE1, flanked by MCS

Example 12. Expression of bovine somatotropin (bST) from modified pBR322-based plasmids containing either homogeneous pMB1, ColE1, or hybrid pMB1/ColE1 origins of replication.

[00115] The host strain LBB427 was used in experiments to determine the effect of hybrid ORI on the expression level of the protein bST. The level of bST protein expression was measured during culture in a fermenter containing a chemically defined inorganic salts and glucose minimal medium (without any antibiotics), with bST synthesis induced by the addition of 50 ppm nalidixic acid at an optical density at 660 nm of 23. The level of bST expression was measured using an HPLC assay with a limit of detection of 1 milligram of bST per liter. The fermentation growth conditions and the HPLC assay are described in Bogosian et al. 1989; as described in that paper, the fermenter contained a minimal medium without any antibiotics.

[00116] An unexpected finding from these experiments was that when the plasmid origin of replication included ColE1-derived origin of replication nucleotide sequences from the AlwNI site to the end of the origin of replication region at coordinate 3148, bST expression was completely abolished. It would not have been predicted that subtle changes in the origin of replication would have such significant effects on the expression of genes elsewhere on the plasmid vector. This finding further illustrates the utility of the present invention, allowing the facile manipulation of plasmid origins of replication for the purpose of testing the effect of such manipulations on heterologous gene expression.

TABLE 6

Quantifying bST protein expression from plasmids containing the bST gene

plasmid	ORI	bST expression mg per liter
pXT757	origin of replication region (positions 1766-3148) taken from pBR322	6400
pXT996	origin of replication region (positions 1766-3148) taken from pBR322, flanked by MCS	5700
pXT985	hybrid origin of replication region with one part of the region (position 1766 to AlwNI restriction site) taken from pBR322, and the other	no detectable expression

	part of the region (from AlwNI restriction site to position 3148) taken from Cole1	
pXT1003	hybrid origin of replication region with one part of the region (position 1766 to AlwNI restriction site) taken from pBR322, and the other part of the region (from AlwNI restriction site to position 3148) taken from Cole1, flanked by MCS	no detectable expression
pXT986	hybrid origin of replication region with one part of the region (position 1766 to AlwNI restriction site) taken from Cole1, and the other part of the region (from AlwNI restriction site to position 3148) taken from pBR322	6200
pXT1002	hybrid origin of replication region with one part of the region (position 1766 to AlwNI) from Cole1, and the other part of the region (from AlwNI to 3148) from pBR322, flanked by MCS	6200
pXT987	origin of replication region (positions 1766-3148) from Cole1	no detectable expression
pXT1004	origin of replication region (positions 1766-3148) from Cole1, flanked by MCS	no detectable expression

Example 13: Construction of pXT1092, using pXT995 as template for inverting the pBR322 origin of replication region with flanking MCS region

[00117] The plasmid pXT995 is a modified pBR322 with a MCS cassette flanking the position of the origin of replication, as described: *Bgl*II-*Nsi*I-*Not*I, origin of replication, *Sac*I-*Spe*I-*Bgl*II. Inverting or flipping the origin of replication region of pXT995 was accomplished by digestion with *Bgl*II, followed by re-ligation. As a result of the re-ligation experiments, eight transformants were obtained and three contained an inverted origin of replication region. One of these transformants was designated pXT1092 (SEQ ID NO: 33) and is shown in Figure 7.

[00118] The plasmid pXT996 is a modified pXT757 with the pBR322 origin of replication flanked by MCS as described: *Bgl*II-*Nsi*I-*Not*I, origin of replication, *Sac*I-*Spe*I-*Bgl*II. Interestingly, numerous attempts to evaluate bST expression of pXT996 with a flipped or inverted origin of replication region by digesting with *Bgl*II followed by re-ligation all failed.

[00119] These results suggested that flipping or inverting of the origin of replication region could not be done in a plasmid like pXT996 containing the bST expression elements. Thus, it appears that with plasmids containing the bST expression elements, the origin of replication region can only be tolerated in one orientation, i.e., that orientation found on the unmodified pBR322 plasmid. Again, this was an unexpected finding, further illustrating the utility of the present invention with regard to manipulation of the structure and orientation of plasmid origins of replication for the purposes of improving the expression of plasmid-borne heterologous genes.

Example 14: Construction of plasmids with origin of replication region from pACYC184.

[00120] The plasmid pACYC184 was derived from the plasmid P15A (Chang and Cohen, 1978). The nucleotide sequence of plasmid pACYC184 is given as **SEQ ID NO:24**. The copy number of P15A was reported to be about 15 (Cozzarelli et al., 1968), and the copy number of pACYC184 has been variously reported to range from about 18 (Chang and Cohen, 1978), or about 30 (Ray and Skurray, 1984), or about 9 (Atlung et al., 1999). The 4245 base pair plasmid, pACYC184, is compatible with pMB1- or ColE1-related plasmids and can therefore be used together with a pMB1- or ColE1-derivative within the same cell. pACYC184 contains: (1) the replicon (*rep*) responsible for the replication of plasmid (this origin of replication is from the plasmid p15A); (2) the *tetR* gene, encoding tetracycline resistance protein; and (3) the *cat* gene, encoding for chloramphenicol acetyl transferase and thus conferring resistance to chloramphenicol.

[00121] The *rop* gene encodes a protein that acts to lower the plasmid copy number when compared to plasmids without the *rop* gene. Therefore, if the *rop* gene could be added to the ORI of a high copy plasmid, one could alter the copy number of the plasmid inside a cell. The plasmid pACYC184 lacks the *rop* gene.

[00122] Figure 8 shows a linear map of the pACYC184 origin of replication containing a single BstZ17I restriction site. As shown in Figure 9, the nucleotide sequence of pACYC184 (nucleotides 568-1407) reveals an origin of replication region with extensive homology to the origin of replication region on pBR322 (nucleotides 2222-3139). On pBR322, the *rop* gene lies just counterclockwise of the BstZ17I site, which is at coordinate 2249 on pBR322 and coordinate 596 on pACYC184. The region of homology between these two origins of replication regions begins just prior to this BstZ17I site.

A. Construction of pXT1094, with the pACYC184 origin of replication flanked by MCS.

[00123] The pACYC184 origin of replication, from coordinates 517 to 1464 (**SEQ ID 24**), was amplified as a 948 bp *NotI*-*SacI* PCR fragment and inserted into the MCS of pXT995. This yielded pXT1094 (**SEQ ID 27**), a plasmid with the pBR322 backbone of the *bla* and *tetR* genes, but with the pBR322 origin of replication replaced by the pACYC184 origin of replication (the plasmid p15A origin of replication), and flanked by MCS.

[00124] The upstream primer (SEQ ID NO:25)

5'-GGCTCAGCAGCGGCCGCGCTGTCCCTCCTGTTCAGCTATTGACGGGG-3'

contains a single nucleotide change in the region near the MCS, outside of the region of homology between the pBR322 and pACYC184 origin of replication regions, eliminating the AlwNI site there by changing the site from CAG-CTA-CTG to CAG-CTA-TTG.

[00125] The downstream primer (SEQ ID NO:26)

5'-GGCTCTGACGAGCTCGGTGCTACATTTGAAGAGATAAATTGCACTGAAATCTAGG-3'

contains a single nucleotide change in the corresponding region near that MCS, eliminating the *Xba*I site there by changing the site from TCTAGA to CCTAGA. The nucleotide sequence of plasmid pXT1094 is given as SEQ ID NO:27.

B. Construction of pXT1091, a composite plasmid with the pACYC184 origin of replication and the pBR322 *rop* gene, flanked by MCS.

[00126] The pACYC184 origin of replication was excised as a *Bst*Z17I-*Sac*I restriction fragment from pXT1094 and inserted into pXT995, creating pXT1091. The nucleotide sequence of pXT1091 is given as SEQ ID NO:28. It should be noted that on pBR322, the *Bst*Z17I site lies between the *rop* gene and the origin of replication region. On pACYC184, the *Bst*Z17I site lies in the same relative position with respect to the origin of replication region, in this case just inside the beginning of the region of homology between the two origin of replication regions (as shown in the alignment of Figure 9 and the pACYC184 origin of replication region shown in Figure 8). The unmodified pACYC184 does not contain a *rop* gene. Thus, the new plasmid pXT1091 has the following genetic elements, in clockwise order, the pBR322 *tetR* gene, the first MCS, the pBR322 *rop* gene, the *Bst*Z17I site, the pACYC184 origin of replication region, the second MCS, and then the pBR322 *bla* gene. The nucleotide sequence of plasmid pXT1091 is given as SEQ ID NO:28.

[00127] This construct was evaluated to determine whether the pBR322 *rop* gene product had any effect on the copy number of a plasmid with the pACYC184 origin of replication region (a p15A origin of replication). The copy number results are shown in Table 7 and are described below.

Example 15: Plasmid copy numbers of pACYC184, pXT1091, and pXT1094

[00128] As an example of the usefulness of the present invention, it can be shown that plasmids modified with hybrid ORI containing the *rop* gene can have their copy numbers in bacteria

altered. The copy numbers for pACYC184, pXT1091, and pXT1094 were determined in strain DH5 α , grown in one set of shake flask cultures grown in L-broth plus the appropriate antibiotic, specifically 10 micrograms per milliliter tetracycline for pACYC184, and 100 micrograms per milliliter ampicillin for pXT1091 and pXT1094. The copy number measurements were made as described above. Table 7 shows the results of this experiment.

TABLE 7

Plasmid copy number during growth in shake flasks with L-broth medium (plus tetracycline or ampicillin)

Plasmid	(hours after inoculation)
	<u>7 hours</u>
pACYC184	85
pXT1091	620
pXT1094	210

[00129] These results suggest that the presence of the *rop* gene, on plasmid pXT1091, actually increased the plasmid copy number rather than lower it (as would have been expected). These findings further illustrate the utility of the present invention with regard to manipulation of the structure plasmid origins of replication for the purposes of improving the expression of plasmid-borne heterologous genes. These results illustrate the utility of the present invention in providing new plasmid vectors with two ranges of plasmid copy numbers, namely plasmid pXT1091 with a plasmid copy number of about 500, and plasmid pXT1094 with a plasmid copy number of about 200.

Example 16: Construction of pXT1110, with the pACYC184 origin of replication flanked by MCS and the pXT757-derived bST protein expression elements, and pXT1109, with the pACYC184 and *rop* gene flanked by MCS and the pXT757-derived bST protein expression elements

[00130] The *NotI-SpeI* fragment from pXT1094, carrying the pACYC184 origin of replication flanked by MCS, was inserted into pXT996, thus replacing the pBR322 origin of replication region, and yielding plasmid pXT1110. The *NotI-SpeI* fragment from pXT1091, carrying the

pACYC184 origin of replication and *rop* gene flanked by MCS, was inserted into pXT996, thus replacing the pBR322 origin of replication region, and yielding plasmid pXT1109 (SEQ IN NO: 34).

[00131] **Example 17. Expression of bST protein from modified pBR322-based plasmids containing either the pMB1, the pACYC184 origins of replication or the pACYC184 plus *rop* origin of replication.**

[00132] As an example of the usefulness of the present invention, it can be shown that plasmids modified with hybrid ORI can alter the level of expression of a target protein. The host strain LBB427 was used in experiments to determine the effect of hybrid ORI on the expression level of the protein bST (bovine somatotrophin). The level of bST protein expression was measured during culture in a fermenter containing a chemically defined inorganic salts and glucose minimal medium (without any antibiotics), with bST protein synthesis induced by the addition of 50 ppm nalidixic acid at an optical density at 660 nm of 2.3. The level of bST protein expression was measured using an HPLC assay with a limit of detection of 1 milligram of bST per liter. The fermentation growth conditions and the HPLC assay are described in Bogosian *et al.* 1989; as described in that paper, the fermenter contained a minimal medium without any antibiotics.

Table 8

Expression of bST protein from plasmids with the pACYC184 origins of replication or pACYC184 plus *rop* origin of replication

<u>plasmid</u>	<u>ori</u>	<u>bST expression mg per liter</u>
pXT757	pBR322 (pMB1)	6400
pXT1109	pACYC184 plus <i>rop</i>	2700
pXT1110	pACYC184	4700

[00133] These results suggest that high plasmid copy numbers have a detrimental effect on this particular bST protein expression system. These findings further illustrate the utility of the

present invention with regard to manipulation of the structure plasmid origins of replication for the purposes of improving the expression of plasmid-borne heterologous genes.

Example 18. Construction of plasmid pXT1007, with a *cer* site

[00134] The *cer* protein typically increases the stability of inheritance of a plasmid. Therefore, it may be useful to include the *cer* gene on a plasmid in order to increase the rate of retention of the plasmid within a cell. The present invention allows for the inclusion of the *cer* gene in a hybrid ORI, thereby demonstrating the advantage of being able to tailor the ORI of a plasmid to suit one's needs.

[00135] The region of ColE1 carrying the *cer* site, and some flanking nucleotide sequence, was isolated as a 357 bp NsiI-NotI PCR fragment prepared from ColE1 plasmid DNA, and inserted into the MCS on pXT996 to yield the plasmid pXT1007. The nucleotide sequence of the NsiI-NotI fragment was determined, and is shown in Figure 10 (SEQ ID 32). The nucleotide sequence given in Figure 10 is different from that found in the Genbank nucleotide sequence of ColE1, as more recent nucleotide sequencing of the plasmid ColE1 by the inventors has shown differences with the Genbank nucleotide sequence; the nucleotide sequence given here is believed by the inventors to be the correct nucleotide sequence of the *cer* site on the plasmid ColE1. The transcribed region giving rise to the RNA molecule RCD is underlined (Patient and Summers, 1993).

Example 19. Stability of plasmids pXT757 (no *cer* site) and pXT1007 (with a *cer* site)

[00136] The stability of inheritance of strains LBB427 [pXT757] and LBB427 [pXT1007] were studied by culturing in LB without any antibiotics. The procedure was to start with a fresh overnight culture grown in LB plus ampicillin and tetracycline (where 100% of the cells would contain the plasmid) and sub-culture in LB without any antibiotics. For each sub-culture, 10 microliters of the previous full-density culture were transferred to 10 ml of LB. This is a 1000-fold dilution, requiring the culture to grow for 10 generations to reach full density again. That is, since $2^{10} = 1024$, 10 doublings of a 1000-fold diluted culture would be required for the culture to increase about 1000-fold back to full density. Since these *E. coli* strains grow in LB at 37°C with a doubling time of about 20 minutes, only a little over 3 hours of exponential growth would be required for the 10 doublings. To allow for any lag time, the sub-cultures were grown for at least 8 hours. The practice was to start a sub-culture in the morning, grow it for 8 hours, then

sub-culture again for overnight growth. Thus, the passage of 20 generations was achieved in a 24 hour period. At the end of each ten generation growth cycle, the full density cultures were diluted and plated on LB and LB Amp to determine the percentage of cells that retained the plasmid.

Table 9

Percent of cells retaining the plasmid after successive generations

<u>Generations</u>	<u>pXT757(- cer)</u>	<u>pXT1007 (+cer)</u>
0	100	100
10	89	95
20	92	100
30	67	97
40	33	94
50	24	100
60	21	99
70	3.5	95
80	2.2	96
90	1.0	100
100	0.34	91

[00137] It is apparent that the addition of the *cer* gene to the pXT757 plasmid has improved the retention rate of the plasmid within the LBB427 cells.

Example 20. Expression of bST from plasmids with the *cer* site

[00138] The host strain, LBB427, was used in experiments to determine the effect of hybrid ORI on the expression level of the protein bST. The level of bST protein expression was measured during culture in a fermenter containing a chemically defined inorganic salts and glucose minimal medium (without any antibiotics), with bST protein synthesis induced by the addition of 50 ppm nalidixic acid at an optical density at 660 nm of 23. The level of bST protein expression was measured using an HPLC assay with a limit of detection of 1 milligram of bST per liter. The fermentation growth conditions and the HPLC assay are described in Bogosian *et al.* 1989; as described in that paper, the fermenter contained a minimal medium without any antibiotics.

Table 10**Influence of *cer* site on bST expression**

<u>Strain and plasmid</u>	<u>fermentation</u>	<u>bST expression mg per liter</u>	
LBB427 [pXT1007]	Run 1	6300	
	Run 2	6300	avg. = 6300

[00139] As shown in Table 6, bST protein expression from pXT757 (lacking the *cer* site) was about 6400 mg per liter. The results here indicate that addition of the *cer* site to the origin of replication in pXT757 (thereby creating the plasmid pXT1007) did not lead to increased expression of bST protein. The lack of any beneficial effect of the increased plasmid retention associated with the addition of the *cer* site may reflect the fact that the fermentation culture was grown for a low number of generations in the absence of antibiotic selection for plasmid retention. Under the fermentation culture conditions used here, the inoculum was grown in L-broth plus antibiotics, and thus 100% of the cells in the inoculum would contain plasmid. However, after inoculation of the fermenter vessel, there were only about 7-8 generations of growth in the fermentation medium in the absence of antibiotics. It would be expected that there would be no detectable loss of plasmid-bearing cells, even without the *cer* site, in only 7-8 generations. Indeed, as shown in Table 9, pXT757 was retained in over 90% of the cells for at least 20 generations.

Example 21. Construction of pXT1221, a composite pBR322 containing a *cer* site and origin of replication flanked by MCS

[00140] The NsiI-SpeI fragment of pXT1007 was inserted into pXT995 (which is pBR322 with the origin of replication flanked by MCS) to yield pXT1221 as shown in Figure 11. The *cer* site and pBR322 origin of replication arrangement on pXT1221 with respect to the MCS is:

[00141] BglII – NsiI – *cer* – NotI – pBR322 origin of replication – SacI – SpeI – BglII

[00142] The stability of DH5 α [pXT1221] was tested as described above for plasmids with and without the *cer* site, the results shown in Table 11. Remarkably, the plasmid stability was virtually unchanged for greater than 300 generations for constructs containing the *cer* site.

Table 11**Percent of cells retaining the plasmid (+/- *cer* site) after successive generations**

<u>Generations</u>	<u>pXT757 (- <i>cer</i>)</u>	<u>pXT1007 (+ <i>cer</i>)</u>	<u>pXT1221 (+ <i>cer</i>)</u>
0	100	100	100
10	89	95	100
20	92	100	100
30	67	97	100
40	33	94	100
50	24	100	100
60	21	99	85
70	3.5	95	100
80	2.2	96	100
90	1.0	100	100
100	0.34	91	98
110			95
120			97
130			100
140			100
150			98
160			100
170			100
180			83
190			100
200			100
250			98
300			100
350			95

[00143] This finding indicates that the inclusion of a *cer* site on a plasmid, an addition made straightforward by the present invention, would be worthy of consideration for culture conditions requiring high plasmid retention for an extended number (greater than 20) of generations of growth in the absence of antibiotic selection.

[00144] As the preceding examples have illustrated, the present invention enables the construction of a variety of new types of plasmid vectors with modified origins of replication. The utility of the present invention is partially illustrated by the construction of new plasmid vectors with a wide range of plasmid copy numbers, ranging from 10 to 500 plasmid copies per cell. The utility of the present invention is also illustrated by the ease with which plasmids could be constructed containing the *cer* site and exhibiting increased plasmid retention during prolonged growth (>20 generations) in the absence of antibiotic selections. Such modified plasmids will also provide

useful cloning tools that allow for regulation of the level of expression of desired or target gene products.

[00145] Based on the examples in the specification, one should be able to create useful hybrid ORI that can alter the copy number of plasmid. One should also be able to create exchangeable ORI or ORI that can have genetic elements added in order to alter the characteristics of the plasmid. With the aid of the present invention, one should be able to customize a plasmid to suit one's needs.

[00146] All of the compositions and/or methods and/or processes and/or apparatus disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and/or apparatus and/or processes and in the steps or in the sequence of steps of the methods described herein. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. The scope of the claims should not be limited by the preferred embodiments set forth herein, but should be given the broadest interpretation consistent with the description as a whole.

REFERENCES

- [00147] Atlung, T., B. B. Christensen, and F. G. Hansen. 1999. Role of the Rom protein in copy number control of plasmid pBR322 at different growth rates in *Escherichia coli* K12. *Plasmid* 41: 110-119.
- [00148] Blakely, G., G. May, R. McCulloch, L.K. Arciszewska, M. Burke, S.T. Lovett, and D.J. Sherratt. 1993. Two related recombinases are required for site-specific recombination at *dif* and *cer* in *Escherichia coli* K-12. *Cell* 75: 351-361.
- [00149] Bogosian, G., B.N. Violand, E.J. Dorward-King, W.E. Workman, P.E. Jung, and J.F. Kane. 1989. Biosynthesis and incorporation into protein of norleucine by *Escherichia coli*. *J. Biol. Chem.* 264: 531-539.
- [00150] Bogosian, G., J.P. O'Neil, and K.C. Terlesky. DNA construct for regulating the expression of a polypeptide coding sequence in a transformed bacterial host cell. United States Patent 6,617,130
- [00151] Bogosian, G., J.P. O'Neil, and N.D. Aardema. Recombinant DNA vectors for expression of somatotropins. United States Patent 6,828,124
- [00152] Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L. and Boyer, H.W. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system, *Gene* 2: 95-113.
- [00153] Chang, A. C., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* 134: 1141-1156.
- [00154] Colloms, S.D., P. Sykora, G. Szatmari, and D.J. Sherratt. 1990. Recombination at *ColE1 cer* requires the *Escherichia coli xerC* gene product, a member of the lambda integrase family of site-specific recombinases. *J. Bacteriol.* 172: 6973-6980.
- [00155] Covarrubias, L., Cervantes, L., Covarrubias, A., Soberon, X., Vichido, I., Blanco, A., Kupersztoch-Portnoy, Y.M. and Bolivar, F. 1981. Construction and characterization of new cloning vehicles. V. Mobilization and coding properties of pBR322 and several deletion derivatives including pBR327 and pBR328, *Gene* 13: 25-35.
- [00156] Cozzarelli, N. R., R. B. Kelly, and A. Kornberg. 1968. A minute circular DNA from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 60: 992-999.
- [00157] Funnell, B.E., and G.J. Phillips 2004. *Plasmid biology*. American Society for Microbiology Press, Washington, DC
- [00158] Guhathakurta, A., and D. Summers. 1995. Involvement of ArgR and PepA in the pairing of *ColE1* dimer resolution sites. *Microbiology* 141: 1163-1171.

- [00159] Guhathakurta, A., I. Viney, and D. Summers. 1996. Accessory proteins impose site selectivity during ColE1 dimer resolution. *Mol. Microbiol.* 20: 613-620.
- [00160] Hodgman, T.C., H. Griffiths, and D.K. Summers. 1998. Nucleoprotein architecture and ColE1 dimer resolution: a hypothesis. *Mol. Microbiol.* 29: 545-558.
- [00161] Horton, R.M., H.D. Hunt, S.N. Ho, J.K. Pullen, and L.R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* 77: 61-68.
- [00162] Patient, M.E., and D.K. Summers. 1993. ColE1 multimer formation triggers inhibition of *Escherichia coli* division. *Mol. Microbiol.* 9: 1089-1095.
- [00163] Peden, K.W., Revised sequence of the tetracycline-resistance gene of pBR322. 1983. *Gene* 22: 277-280.
- [00164] Ray, A., and R. Skurray. 1984. Stabilization of the cloning vector pACYC184 by insertion of F plasmid leading region sequences. *Plasmid* 11: 272-275.
- [00165] Sambrook et al., 1989. *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [00166] Stirling, C.J., S.D. Colloms, J.F. Collins, G. Szatmari, and D.J. Sherratt. 1989. *xerB*, an *Escherichia coli* gene required for plasmid ColE1 site specific recombination is identical to *pepA*, encoding aminopeptidase A, a protein with substantial similarity to bovine lens leucine aminopeptidase. *EMBO J.* 8: 1623-1627.
- [00167] Stirling, C.J. G. Szatmari, G. Stewart, M.C.M. Smith, and D.J. Sherratt. 1988. The arginine repressor is essential for plasmid stabilising site-specific recombination at the ColE1 *cer* locus. *EMBO J.* 7: 4389-4395.
- [00168] Summers, D.K. 1989. Derivatives of ColE1 *cer* show altered topological specificity in site-specific recombination. *EMBO J.* 8: 309-316.
- [00169] Summers, D.K. 1991. The kinetics of plasmid loss. *TIBTECH* 9: 273-278.
- [00170] Summers, D.K. 1996. The biology of plasmids. Blackwell Science Ltd., London
- [00171] Summers, D.K., and D.C.D. Rowe. 2001. Methods and means relating to quiescent cells and uses thereof. United States Patent 6,190,867.
- [00172] Summers, D.K., and D.J. Sherratt. 1988. Resolution of ColE1 dimers requires a DNA sequence implicated in the three-dimensional organization of the *cer* site. *EMBO J.* 7: 851-858.

- [00173] Summers, D.K., C.W.H. Beton, and H.L. Withers. 1993. Multicopy plasmid instability – the dimer catastrophe hypothesis. *Mol. Microbiol.* 8: 1031-1038.
- [00174] Summers, D., S. Yaish, J. Archer, and D. Sherratt. 1985. Multimer resolution systems of ColE1 and ColK: localisation of the crossover site. *Mol. Gen. Genet.* 201: 334-338.
- [00175] Sutcliffe, J.G. 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. *Proc. Natl. Acad. Sci. U.S.A.*, 75: 3737-3741.
- [00176] Sutcliffe, J.G. 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. *Cold Spring Harb. Symp. Quant. Biol.*, 43: 77-90.
- [00177] Watson, N. 1988. A new revision of the sequence of plasmid pBR322. *Gene* 70: 399-403.

The embodiments of the present invention for which an exclusive property or privilege is claimed are defined as follows:

1. A hybrid origin of replication comprising nucleotide sequences from origins of replication from at least two different plasmids, wherein the hybrid origin of replication comprises at least 200 contiguous nucleotides from an origin of replication of a first plasmid selected from the group consisting of pBR322, pACYC184, and ColE1; and at least 200 contiguous nucleotides from an origin of replication of a second plasmid selected from the group consisting of pBR322, pACYC184, and ColE1.
2. The hybrid origin of replication of claim 1 comprising at least 200 contiguous nucleotides from nucleotide sequences from a pBR322 origin of replication.
3. The hybrid origin of replication of claim 1 comprising at least 200 contiguous nucleotides from nucleotide sequences from a ColE1 origin of replication.
4. The hybrid origin of replication of claim 1 comprising at least 200 contiguous nucleotides from nucleotide sequences from a pACYC184 origin of replication.
5. The hybrid origin of replication of claim 1 comprising at least 200 contiguous nucleotides from nucleotide sequences from a pACYC184 origin of replication and at least 200 contiguous nucleotides from a pBR322 origin of replication.
6. The hybrid origin of replication of claim 1 comprising at least 200 contiguous nucleotides from nucleotide sequences from a pBR322 origin of replication and at least 200 contiguous nucleotides from a ColE1 origin of replication.
7. The hybrid origin of replication of claim 1 comprising at least 200 contiguous nucleotides from nucleotide sequences from a ColE1 origin of replication and at least 200 contiguous nucleotides from a pACYC184 origin of replication.
8. The hybrid origin of replication of claim 1 comprising at least 250 contiguous nucleotides from nucleotide sequences from a pBR322 origin of replication.

9. The hybrid origin of replication of claim 1 comprising at least 250 contiguous nucleotides from nucleotide sequences from a ColE1 origin of replication.
10. The hybrid origin of replication of claim 1 comprising at least 250 contiguous nucleotides from nucleotide sequences from a pACYC184 origin of replication.
11. The hybrid origin of replication of claim 1 comprising at least 250 contiguous nucleotides from nucleotide sequences from an origin of replication from a plasmid selected from the group consisting of pBR322, pACYC184, and ColE1.
12. The hybrid origin of replication of claim 1 comprising at least 250 contiguous nucleotides from nucleotide sequences from a pACYC184 origin of replication and at least 250 contiguous nucleotides from a pBR322 origin of replication.
13. The hybrid origin of replication of claim 1 comprising at least 250 contiguous nucleotides from nucleotide sequences from a pBR322 origin of replication and at least 250 contiguous nucleotides from a ColE1 origin of replication.
14. The hybrid origin of replication of claim 1 comprising at least 250 contiguous nucleotides from nucleotide sequences from a ColE1 origin of replication and at least 250 contiguous nucleotides from a pACYC184 origin of replication.
15. The hybrid origin of replication of claim 1, wherein the hybrid origin of replication is comprised within SEQ NO:7 or 12.
16. The hybrid origin of replication of claim 1, comprising nucleotide sequences from a pBR322 origin of replication and nucleotide sequences from a ColE1 origin of replication.
17. The hybrid origin of replication of claim 16, wherein a portion of sequence between nucleotide positions 1766 to 3148 of pBR322 (SEQ ID NO: 1) is replaced with at least 200 contiguous nucleotides from ColE1 (SEQ ID NO: 6).

18. The hybrid origin of replication of claim 16, comprising nucleotide sequences from position 1766 to an AlwNI restriction site in pBR322 (SEQ ID NO: 1) linked to nucleotide sequences from an AlwNI restriction site to position 3148 in ColE1 (SEQ ID NO: 6).
19. The hybrid origin of replication of claim 16, comprising nucleotide sequences from position 1766 to an AlwNI restriction site in ColE1 (SEQ ID NO: 6) linked to nucleotide sequences from an AlwNI restriction site to position 3148 in a pBR322 (SEQ ID NO: 1).
20. The hybrid origin of replication of claim 1, comprising sequences from a pBR322 origin of replication and sequences from an origin of replication from ColE1 or pACYC184.
21. The hybrid origin of replication of claim 1 comprising nucleotide sequences from a pACYC184 origin of replication and nucleotide sequences from a ColE1 origin of replication.
22. The hybrid origin of replication of any one of claims 1–21, wherein the hybrid origin of replication is flanked on each side by nucleotide sequences coding for at least one cloning site.
23. The hybrid origin of replication of any one of claims 1–21, wherein the hybrid origin of replication is flanked on each side by nucleotide sequences coding for multiple cloning sites.
24. An exchangeable origin of replication cassette comprising the hybrid origin of replication of any one of claims 1–21 flanked on each side by nucleotide sequences coding for at least one cloning site, wherein said cloning site is not within a regulatory or structural coding region.
25. An exchangeable origin of replication cassette comprising the hybrid origin of replication of any one of claims 1–21 flanked on each side by nucleotide sequences coding for multiple cloning sites, wherein said cloning sites are not within a regulatory or structural coding region.
26. The exchangeable origin of replication cassette of claim 24 or 25, wherein said flanking cloning sites comprise restriction endonuclease sites.

27. The exchangeable origin of replication cassette of claim 25, wherein said cloning sites comprise at least one BspEI, BglII, NsiI, NotI, SacI, SpeI or AlwNI restriction endonuclease site.
28. A plasmid comprising the hybrid origin of replication of any one of claims 1–21 flanked by cloning sites, wherein said cloning sites are not within a regulatory or coding sequence.
29. The plasmid of claim 28, wherein the plasmid has a backbone from pBR322, pMB1, p15A, pACYC184, pACYC177, ColE1, pBR3286; p1, pBR26, pBR313, pBR327, pBR328, pPIGDM1, pPVUI, pF, pSC101 or pC101p-157.
30. The plasmid of claim 28, wherein the plasmid has a backbone from pACYC184.
31. The plasmid of claim 28, wherein the plasmid has a backbone from pBR322.
32. The plasmid of claim 28, wherein the plasmid has a backbone from ColE1.
33. The plasmid of claim 28, wherein the plasmid is SEQ ID NO: 22 or 23.
34. The plasmid of claim 28, wherein the hybrid origin of replication comprises nucleotide sequences from a pBR322 origin of replication and nucleotide sequences from a ColE1 origin of replication.
35. The-plasmid of claim 28, wherein the plasmid is SEQ ID NO: 7 or 12.
36. The plasmid of claim 28, wherein the hybrid origin of replication comprises nucleotide sequences from a pACYC184 origin of replication.
37. A bacterial host cell transformed with the plasmid of any one of claims 28 to 36.
38. A method of creating an exchangeable origin of replication cassette within a first plasmid comprising:
 - (a) using primers to amplify an existing hybrid origin of replication within a first plasmid, wherein the primers comprise an annealing region and a region comprising at least one cloning site that corresponds to a cloning site in a second plasmid;

(b) isolating the PCR amplicons, said amplicons containing an origin of replication region, an annealing region and at least one cloning site.

39. A method of exchanging a hybrid origin of replication region between a first plasmid and a second plasmid comprising:

(a) using primers to amplify an existing hybrid origin of replication within a first plasmid, wherein the primers comprise an annealing region and a region comprising at least one closing site that corresponds to a cloning site in a second plasmid;

(b) isolating the PCR amplicons, said amplicons containing an origin of replication region, an annealing region and at least one cloning site;

(c) digesting the PCR amplicons with a restriction enzyme that recognizes the at least one cloning site;

(d) digesting the second plasmid with a restriction enzyme that recognizes the at least one cloning site so as to cut out the existing origin of replication;

(e) ligating the PCR amplicons of the first plasmid into the second plasmid.

40. A method of producing a recombinant protein of interest comprising:

(a) transforming a suitable host cell with a plasmid of any one of claims 28 to 36 containing a gene encoding a recombinant protein of interest, operatively linked to expression control sequences;

(b) growing a culture of said suitable host cell transformed with said plasmid under suitable conditions for expression of said recombinant protein, and

(c) recovering and purifying the protein of interest.

41. A method of producing plasmid DNA in bacteria comprising:

(a) transforming a suitable bacteria strain with a plasmid of any one of claims 28 to 36; and

(b) growing a culture of said bacteria transformed with said plasmid under conditions which allow replication of said plasmid.

42. The hybrid origin of replication of claim 1, wherein nucleotide sequences from a first origin of replication are replaced with nucleotide sequences from a second origin of replication.

43. A method of creating a hybrid origin of replication of claim 1 comprising replacing nucleotides from a first origin of replication selected from the group consisting of pBR322, pACYC184, and ColE1 with nucleotides from a second origin of replication selected from the group consisting of pBR322, pACYC184, and ColE1.

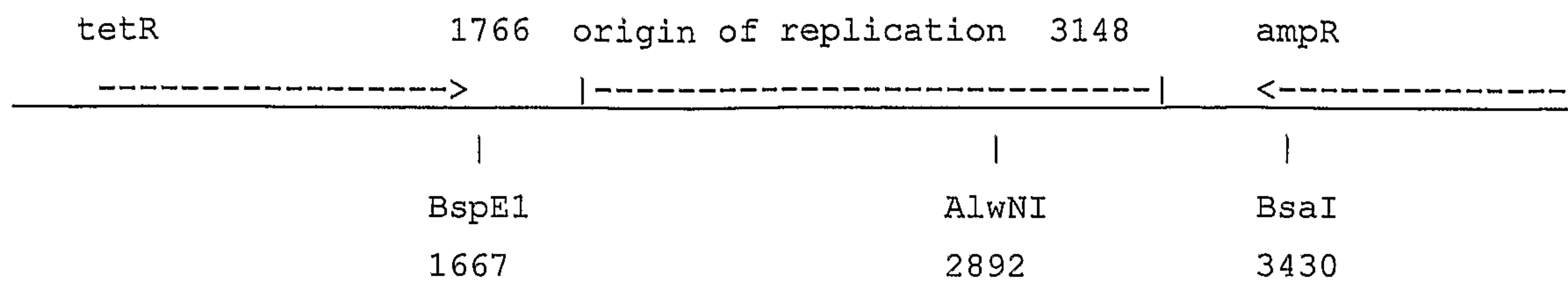


FIG 1

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FIG 2

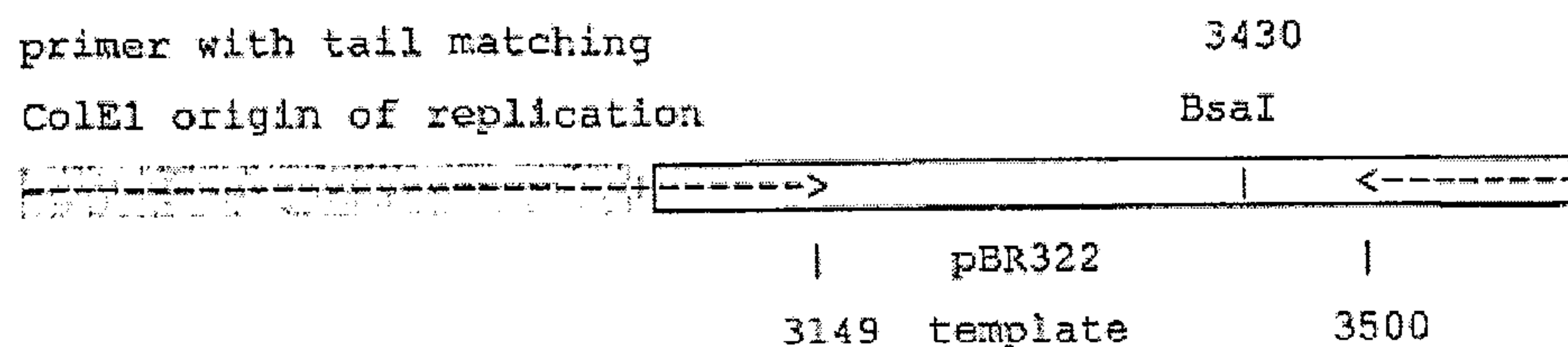


FIG 2A

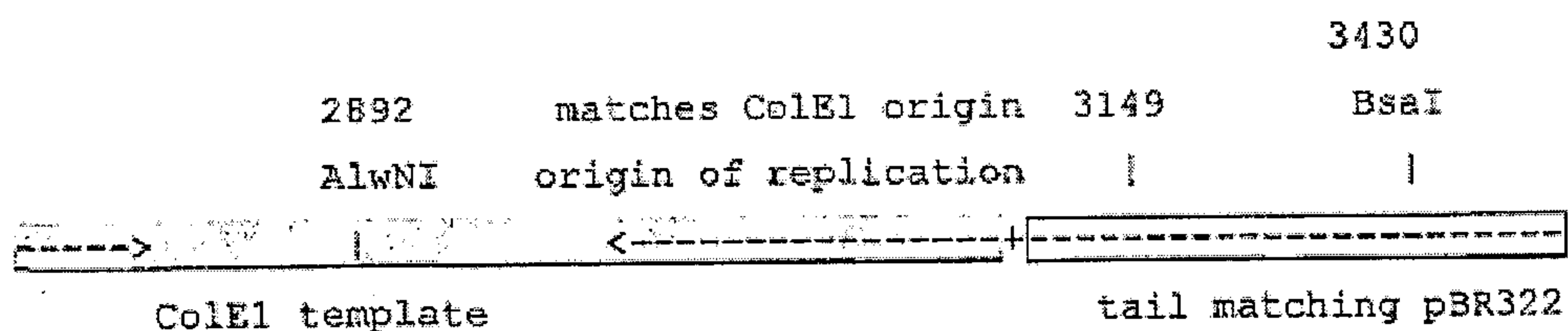


FIG 2B

pXT975

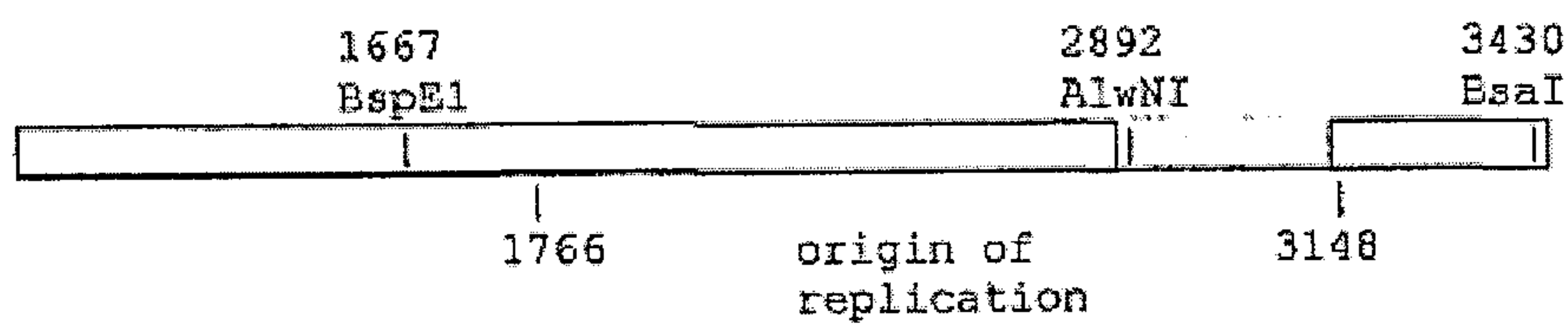


FIG 2C

key: box indicates pBR322 sequences
 grey indicates ColE1 sequences

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FIG 3

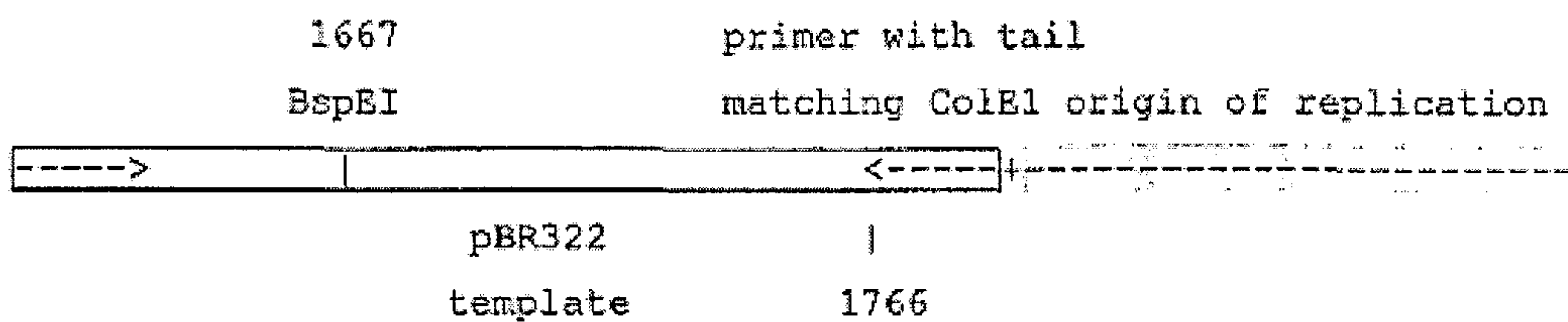


FIG 3A

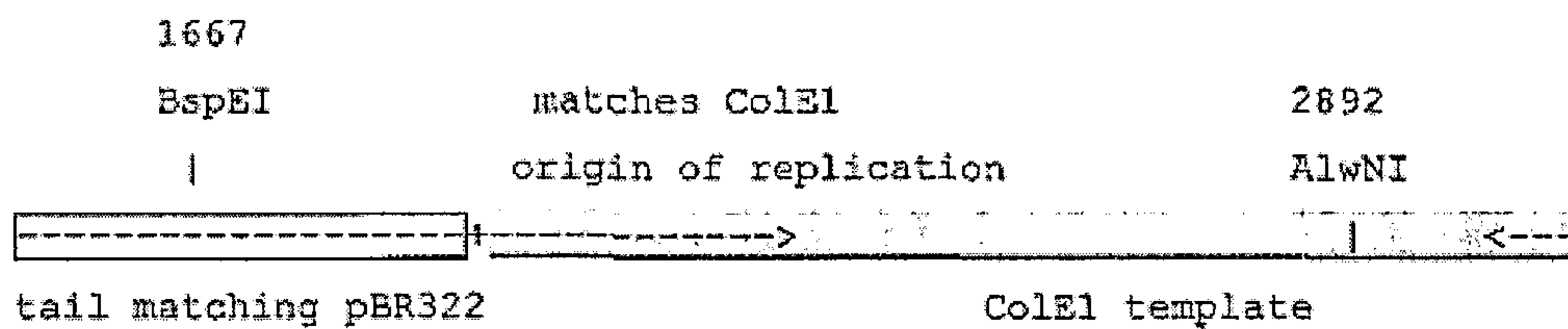


FIG 3B

pXT976

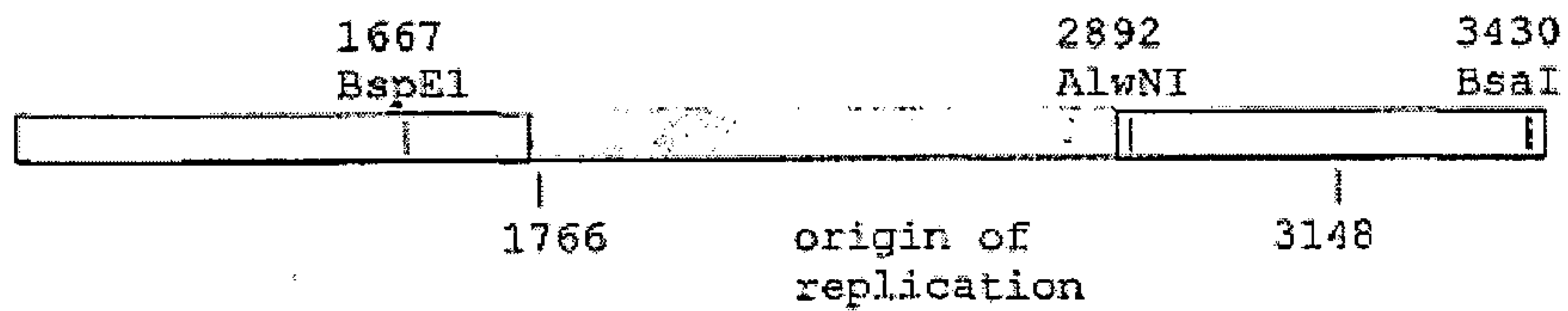
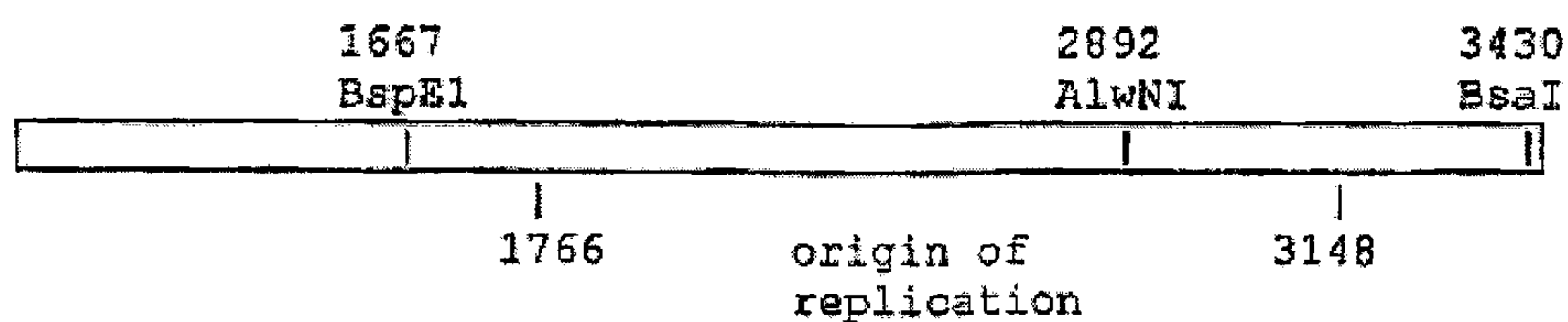
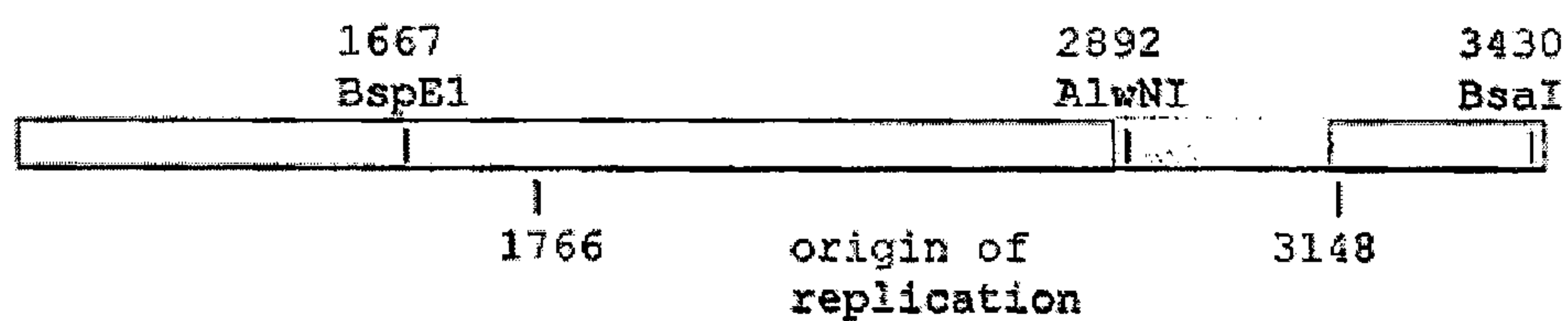
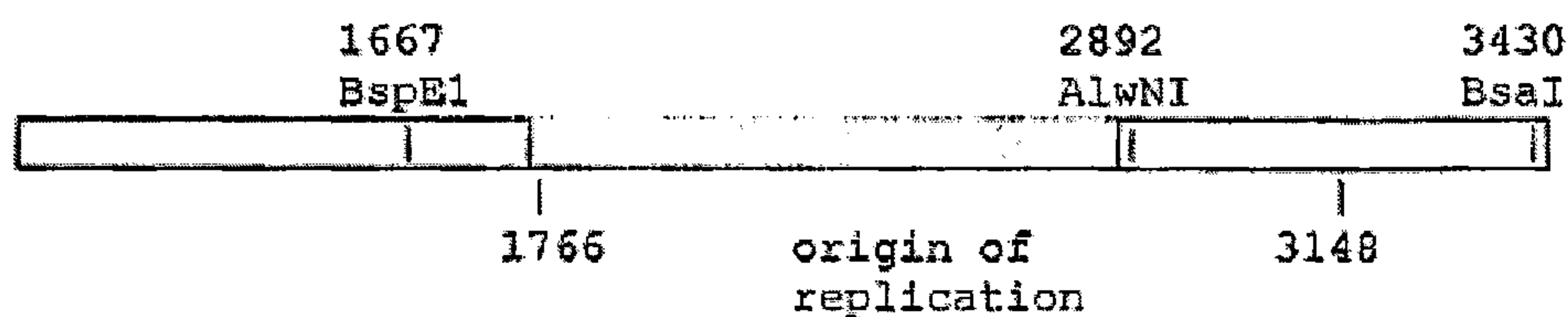
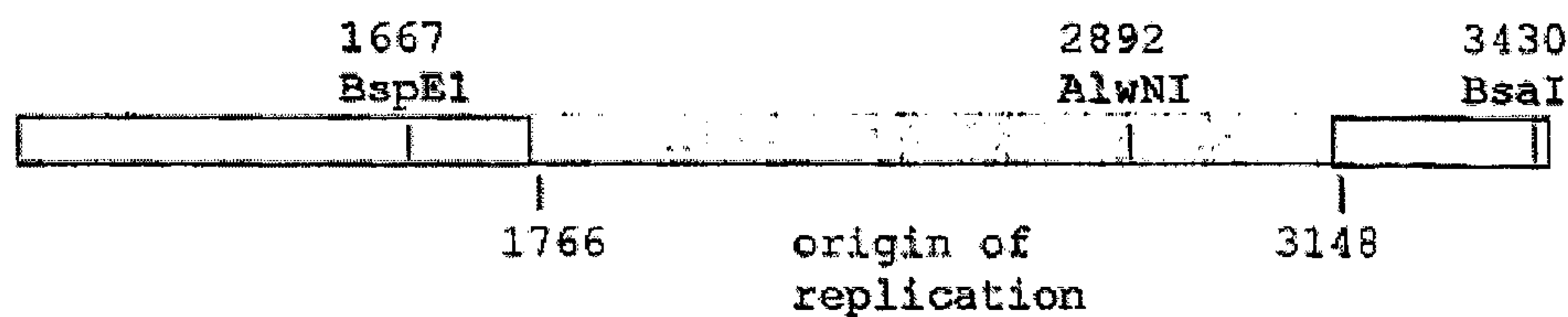


FIG 3C

key: indicates pBR322 sequences
 indicates ColE1 sequences

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pBR322**pXT975****pXT976****pXT977**

key: box indicates sequences derived from the pBR322 origin of replication
grey indicates sequences derived from the ColEI origin of replication

FIG 4

FIG 5

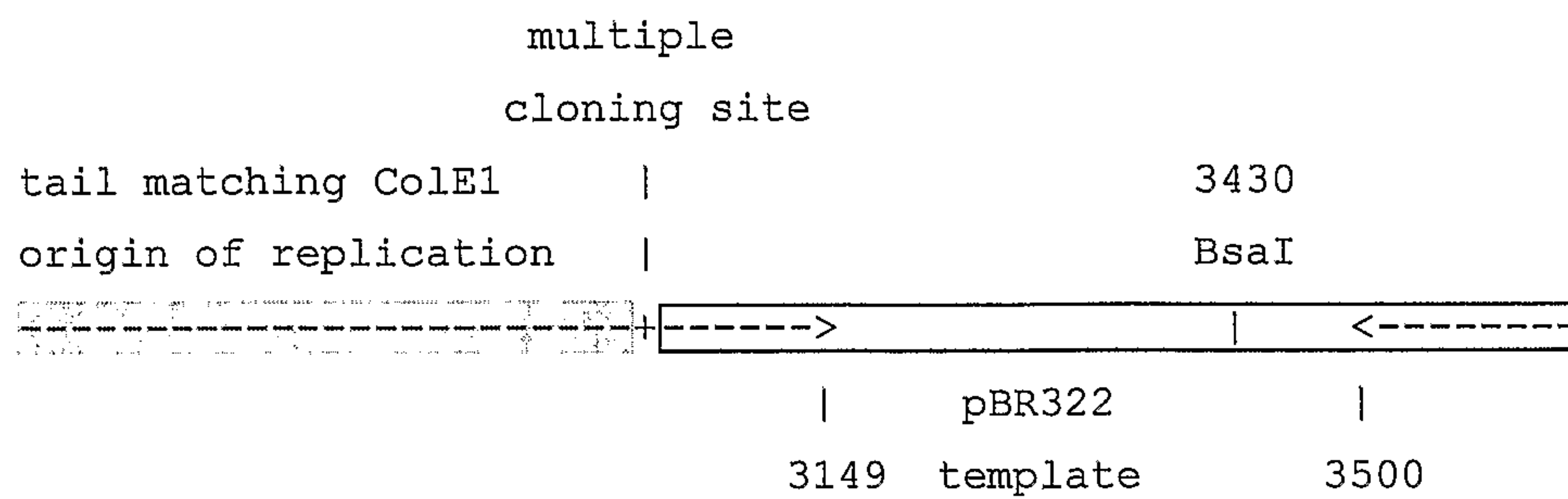


FIG 5A

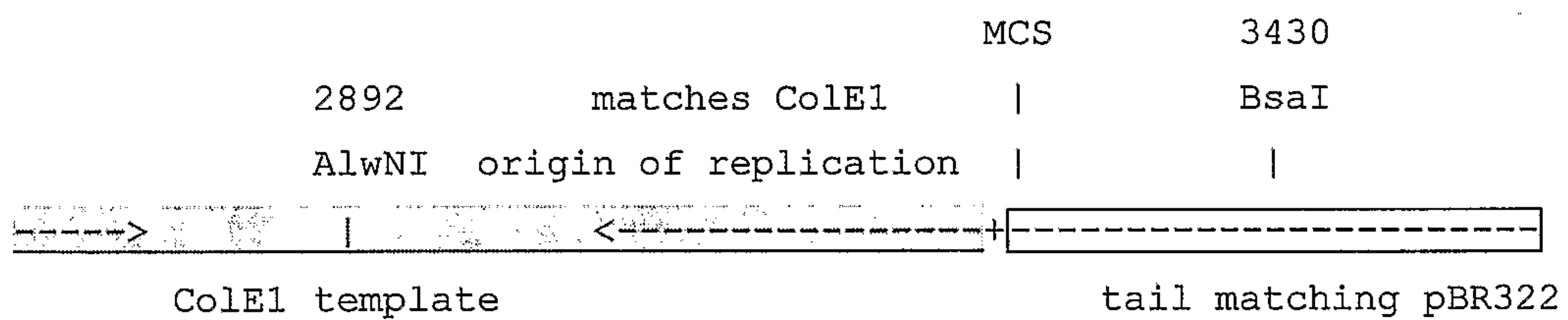


FIG 5B

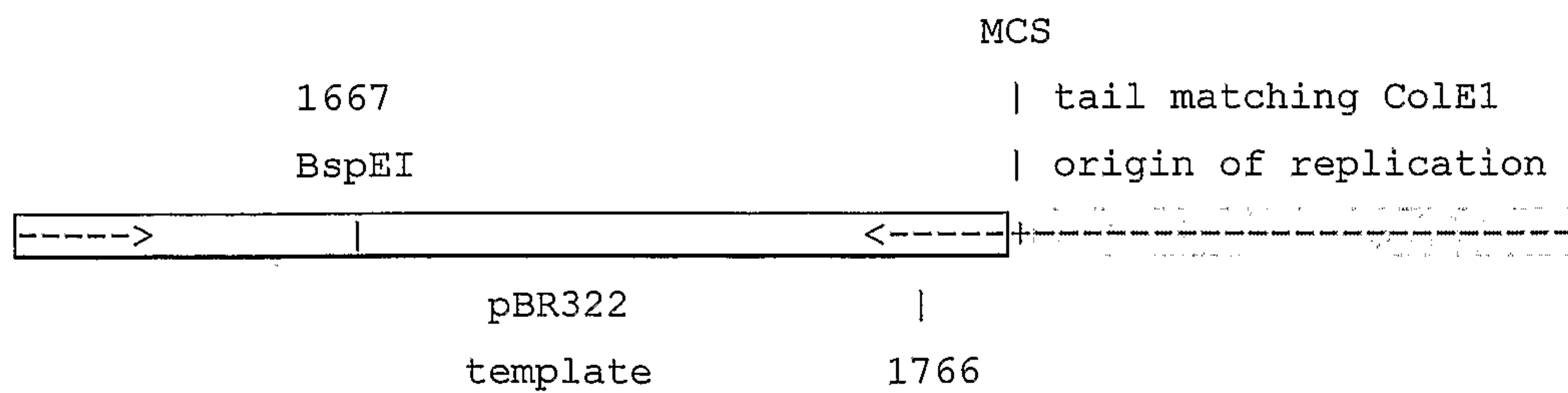


FIG 5C

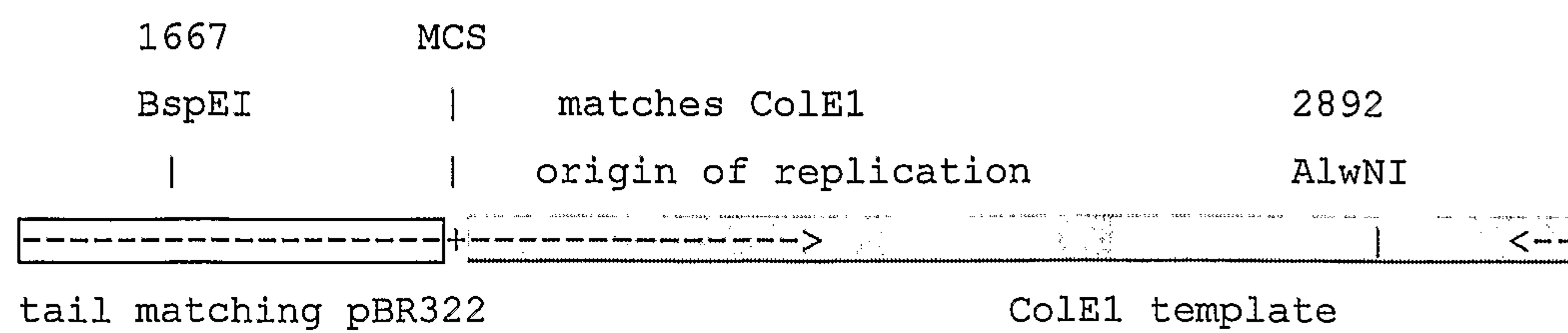


FIG 5D

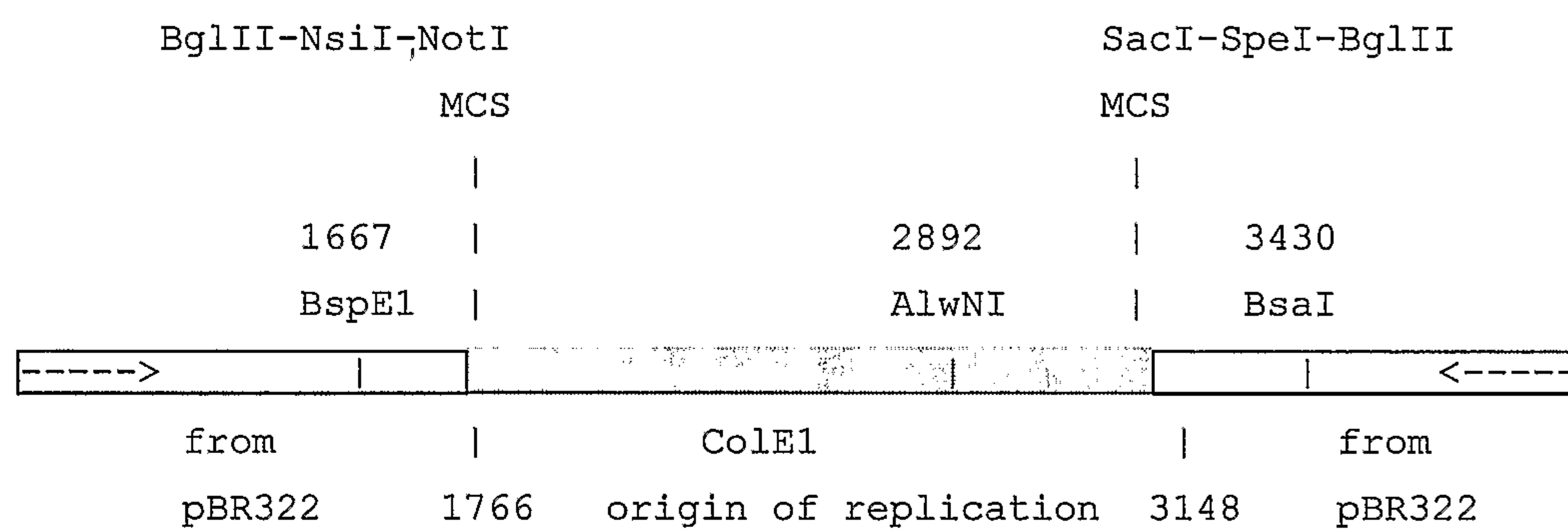


FIG 5E

key: box indicates pBR322 sequences

grey indicates ColE1 sequences

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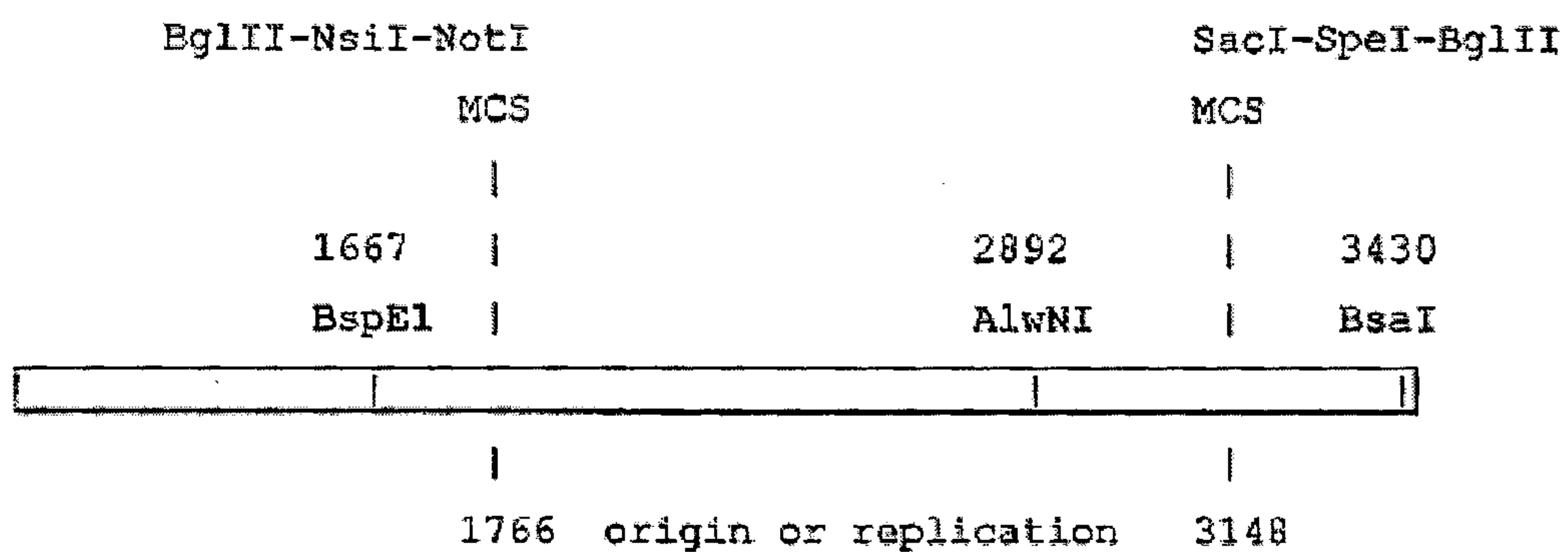
pXT995

FIG 6A

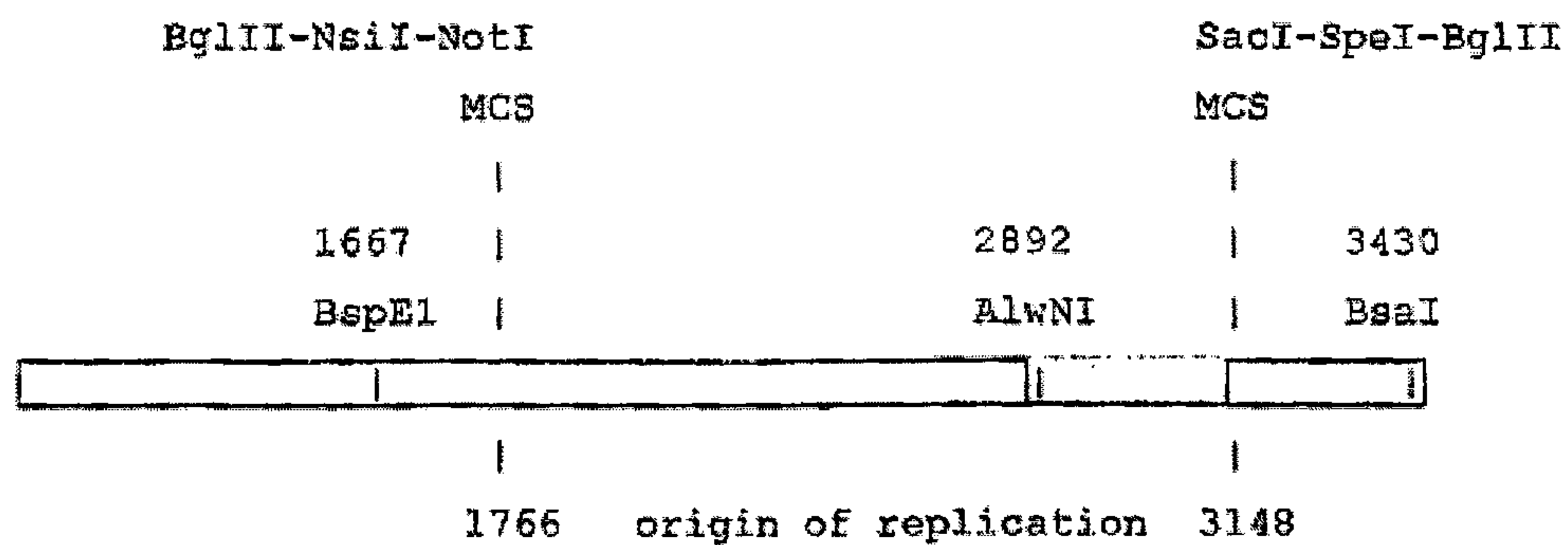
pXT1001

FIG 6B

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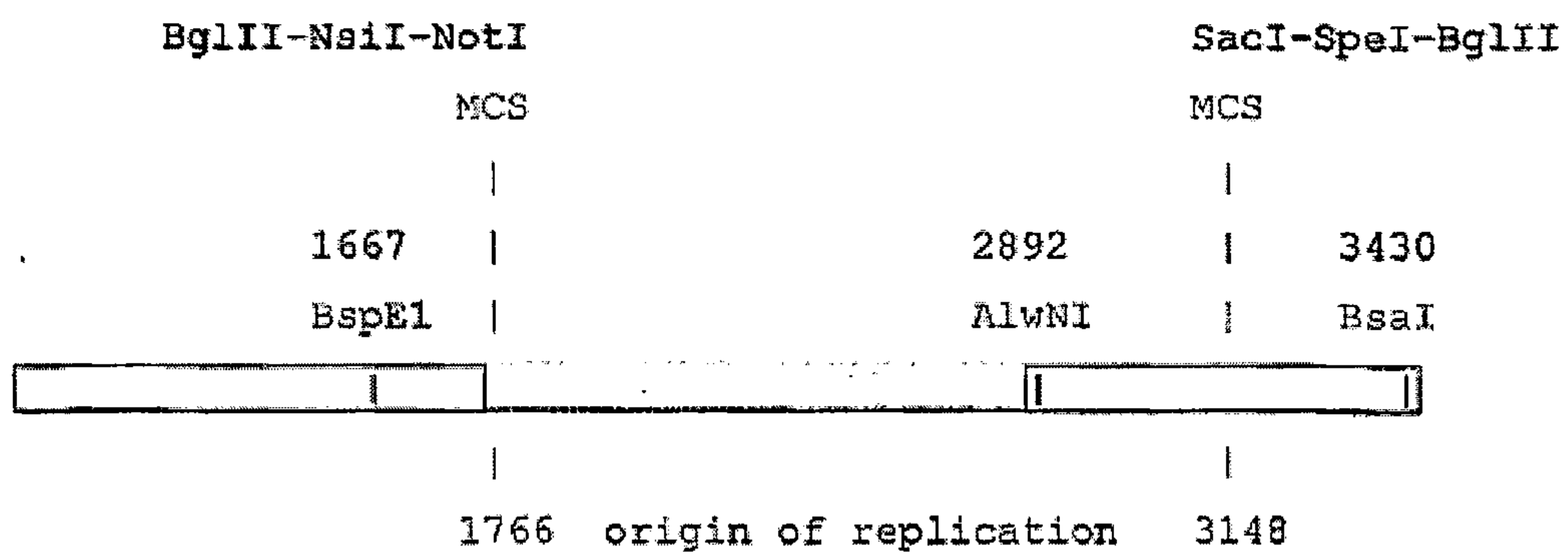
pXT1000

FIG 6C

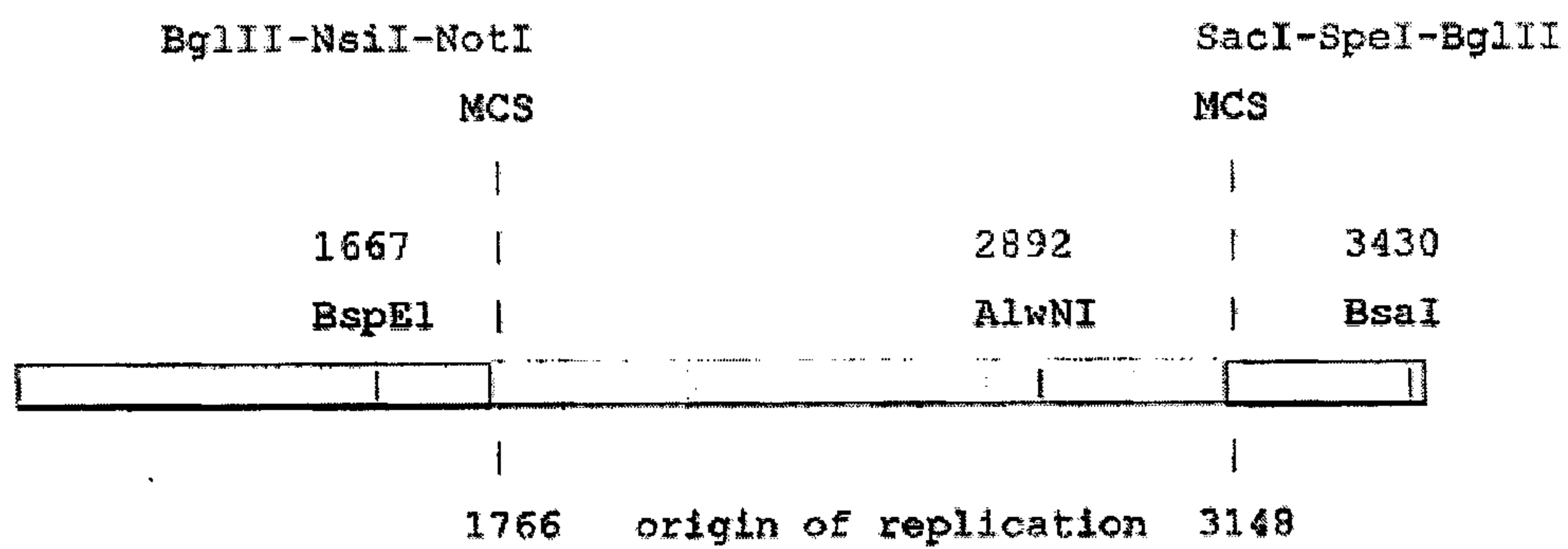
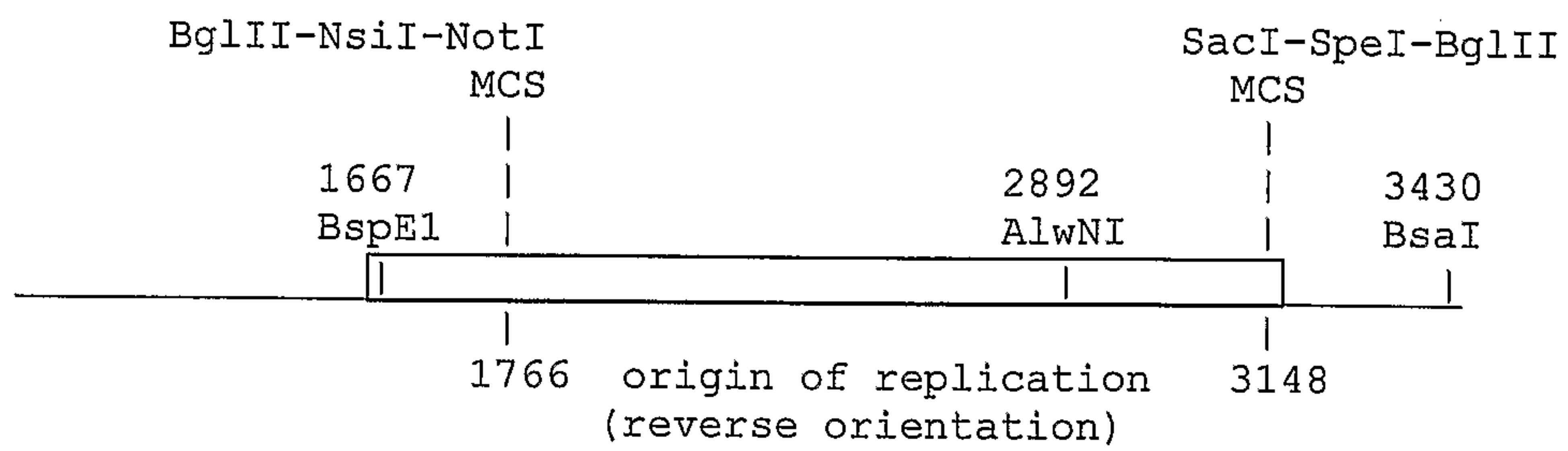
pXT988

FIG 6D

key: indicates pBR322 sequences
 indicates ColE1 sequences

pXT1092**FIG 7**

key: box indicates pBR322 origin of replication sequence

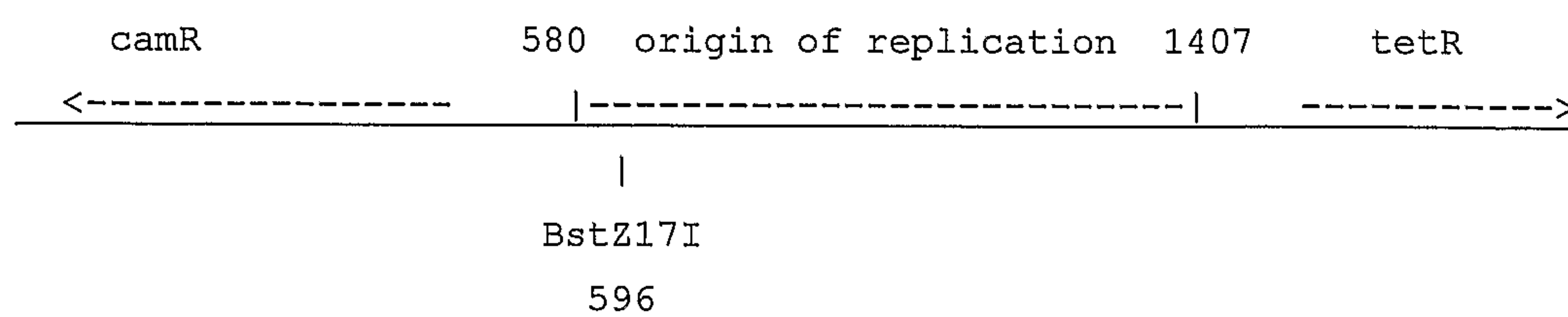


FIG 8

BstZ17I

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2222 CCCAGTCACGT.AGCGATAGCGGAGTGTATACTGGCTTAACTATGCGGCA 2270
      ||      || | |||| ||||| ||||| ||||| ||||| ||||| |
568 CCGCCGGACATCAGCGCTAGCGGAGTGTATACTGGCTT.ACTATGTTGGC 616

2271 TCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATA...CC 2317
      | ||   ||   |||      ||||| ||| ||   | |||| |   |
617 ACTGATGAGGGTGTCAAGTGCTTCATGTGGCAGGAGAAAAAAGGCT 666

2318 GCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCTCTTCCGCTTCCT 2367
      |||| | ||||| || |||| ||   |   ||||   | ||||| |||||
667 GCACCGGTGCGTCAGCAGAATAT.GTGATACAGGATATATTCGCTTCCT 715

2368 CGCTCACTGACTCGCTGCGCTCGGTCGTTTCGGCTGCGGCGAGCGGTATCA 2417
      ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |
716 CGCTCACTGACTCGCTACGCTCGGTCGTTTCGACTGCGGCGAGCGGAAATG 765

2418 GCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGC 2467
      ||| || | || | | |   |
766 GCTTACGAACGGGGCGGAGATTCCT 791 - - - - -

2468 AGGAAGAACATGTGA 2483

2484 GCAAAAGGCCAGCAAAAGGC....CAGGAACCGTAAAAAGGCCGCGTTGC 2529
      | || | ||||| || | |   |||| | || | | |||||
792 GGAAGATGCCAGGAAGATACTTAACAGGGA.AGTGAGAGGGCCGCGGCAA 840

2530 TGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGA 2579
      | ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||
841 AGCCGTTTTTCCATAGGCTCCGCCCCCCTGACAAGCATCACGAAATCTGA 890

2580 CGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATAACCAGGC 2629
      ||||| |||| | ||||| ||||| ||||| ||||| ||||| |||||
891 CGCTCAAATCAGTGGTGGCGAAACCCGACAGGACTATAAAGATAACCAGGC 940

2630 GTTTCCTCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGC 2679
      ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
941 GTTTCCTCCCTGGCGGCTCCCTCGTGCGCTCTCCTGTTCCCTGCCTTTCGGT 990

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FIG 9A

```

2680 TTACCGGATACCTGTCCGCCTTCTCCCTTCGGGAAGCGTGGCGCTTCTCA 2731
      ||||| | | |||| || | | ||   |
991 TTACCGG.TGTCATTCCGCTGTTATGGCCGCGTTTGTCTCA 1030 - - -

```

```

2732 TAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTTCGCTCCAAGC 2781
    |   |   |       |   |   |||||   |   ||||   |||||   |||||
1031 TTCCAC.....GCCTGACACTCAGTTCGGGTAGGCAGTTCGCTCCAAGC 1075

2782 TGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCC 2831
    |||   |||   |||||   |||||   |||||   |||||   |||||   |||||
1076 TGGACTGTATGCACGAACCCCCCGTTCAGTCCGACCGCTGCGCCTTATCC 1125

2832 GGTA ACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACT 2881
    |||||   |||||   |||||   |||||   |||||   |||||   |   |   |||||
1126 GGTA ACTATCGTCTTGAGTCCAACCCGGAAAGACATGCAA AAGCACC ACT 1175


                AlwNI
2882 GGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAG 2917
    |||||   |||||   |||||   |||   |||   ||
1176 GGCAGCAGCCACTGGTAATTGATTTAGAGGAGTTAG 1211


2718 GTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTAC 2963
           |||||   ||   |   ||
- - - - - 1212 TCTTGAAGTCATGCGCCGGTTAA 1234


2964 GGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGT 3013
    |||||   |||   |||||   |||||   |||||   |   |||||
1235 GGCTAAACTGAAAGGACAAGTTTTGGTGACTGCGCTCCTC.CAAGCCAGT 1283

3014 TACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCG 3063
    |||||   |   |||||   |||||   |   |   ||   ||||
1284 TACCTCGGTTCAAAGAGTTGGTAGCTC...AGAGAACCTTCGAAAACCG 1330

3064 CTGGTAGCGGTGGTTTTTTTGTGCA.AGCAGCAGATTACGCGCAGAAA 3112
    |           ||   |||||   ||||   ||   |||   |||||   |||||
1331 CCCTGCAAGGCGGTTTTTTCGTTTTTCAGAGCAAGAGATTACGCGCAGACC 1380

3113 AAAAGGATCTCAAGAAGATCCTTTGAT 3139
    ||||   |||||   |||||   ||   ||   ||
1381 AAAACGATCTCAAGAAGATCATCTTAT 1407

```

FIG 9B

2712

| NsiI

ATGCATGTTATCCCTAGAACGGGAGCGTCAGCCGGAATACAGGAACGCA

CGCTGGATGGCCCTTCGCTGGGATGGTGAAACCATGAAAAATGGCAGCTT

*

CAGTGGATTAAGTGGGGGTAATGTGGCCTGTACCCTCTGGTTGCATAGGT

this G not in Genbank

+1 of RCD |

ATTCATACGGTTAAAATTTATCAGGCGCGATCGCGGCAGTTTTTCGGGTG

|
A in GenbankGTTTGTGCCATTTTTACCTGTCTGCTGCCGTGATCGCGCTGAACGCGTT

end RCD

TTAGCGGTGCGTACAATTAAGGGATTATGGTAAATCCACTTACTGTCTGC

TaqI

|

CCTCGTAGCCATCGAGATAAACCGCACGAAATCGTGTCAGCCAGCAGCCG

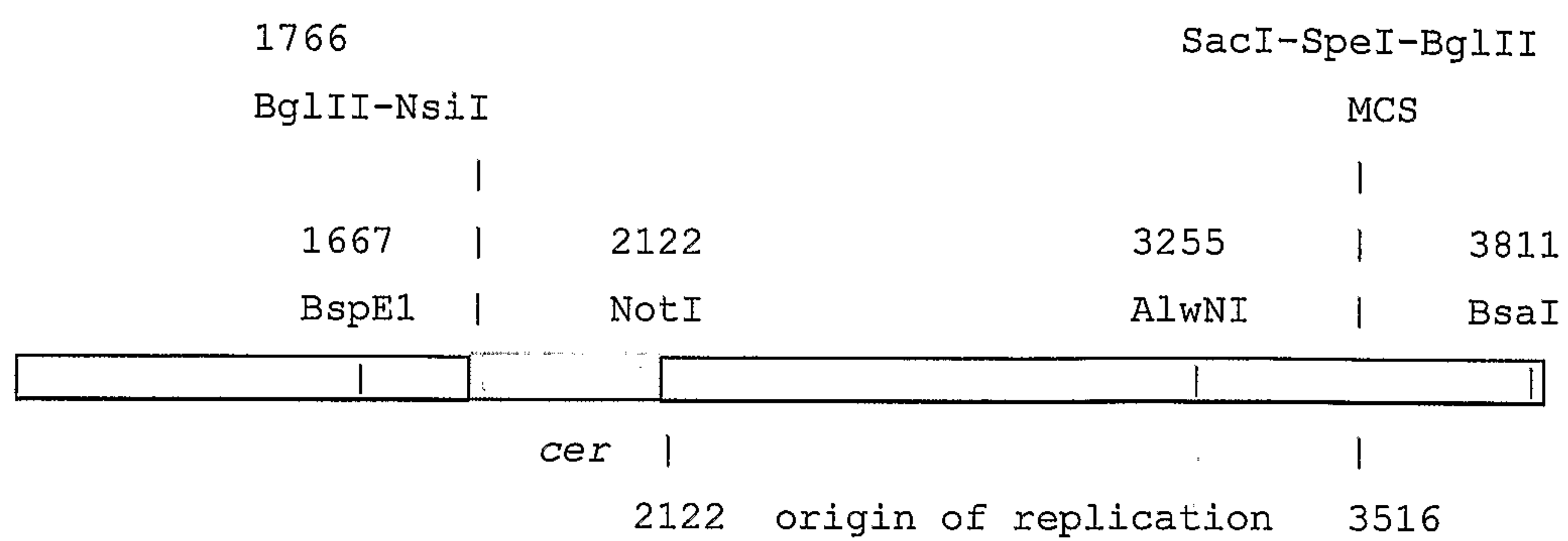
NotI

CGGCCGC

|

3068

FIG 10

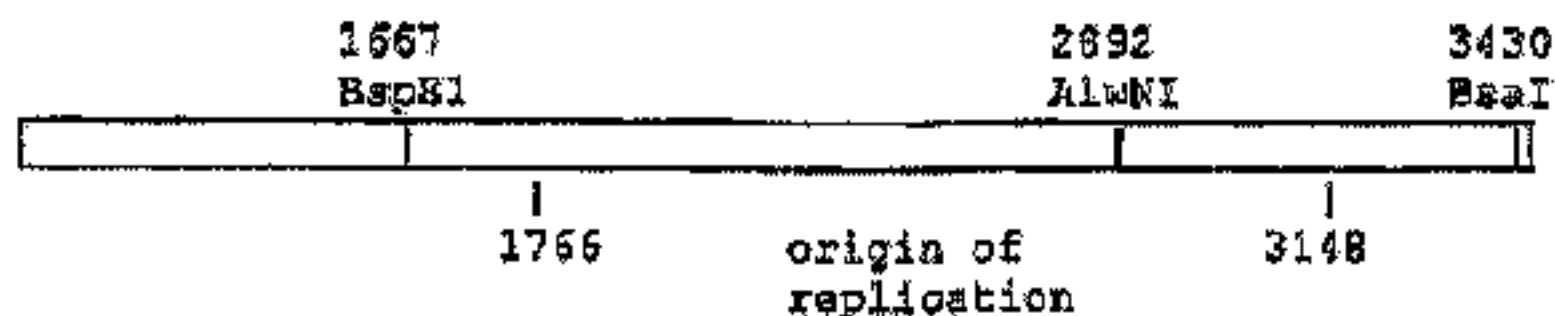


key: indicates pBR322 origin of replication sequences

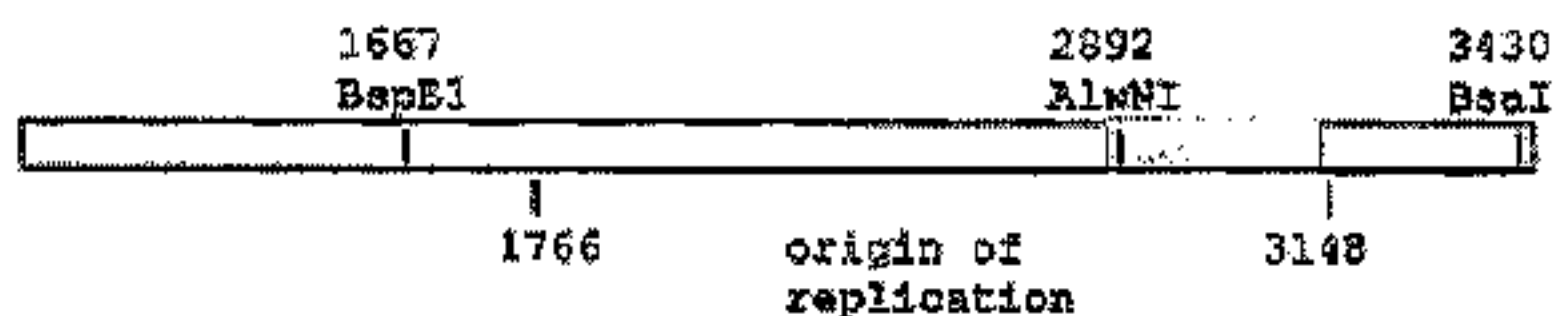
 indicates *cer* site sequences

FIG 11

pBR322



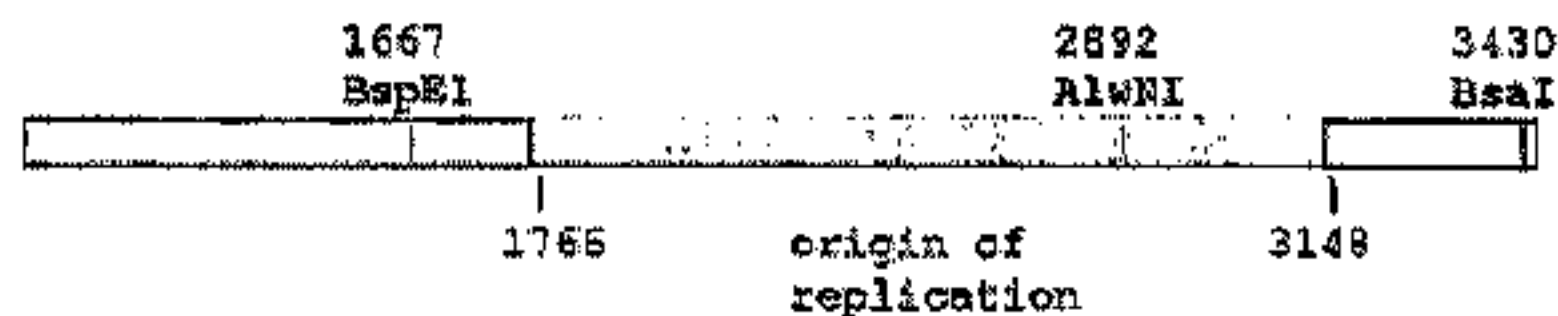
pXT975



pXT976



pXT977



key: indicates sequences derived from the pBR322 origin of replication
 indicates sequences derived from the ColEI origin of replication